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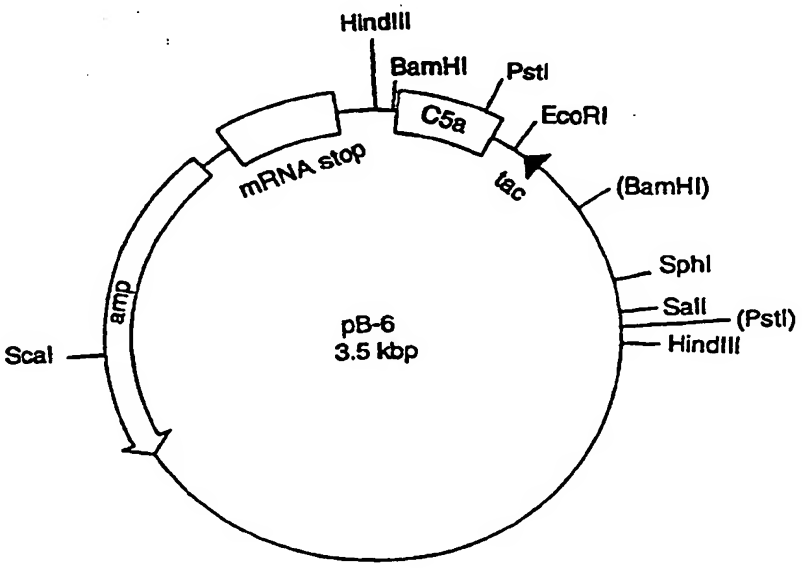
(54) Title: C5a RECEPTOR ANTAGONISTS HAVING SUBSTANTIALLY NO AGONIST ACTIVITY AND METHODS FOR PREPARATION

(57) Abstract

Disclosed are polypeptide analogues or derivatives of human C5a which are C5a receptor antagonists that exhibit substantially no analphylatoxin or agonist activity, and derivatives of the analogues, and dimeric forms of the analogues or derivatives. DNA molecules encoding the polypeptides and methods of making the analogues are also provided. Pharmaceutical formulations containing a C5a analogue or derivative, are used therapeutically in the treatment of C5a-mediated diseases and inflammatory conditions in mammals, and prophylactically to prevent or reduce inflammation caused by an event which causes inflammation or aggravates an existing inflammatory condition, respectively. Further disclosed are antibodies specific to the C5a analogues, derivatives thereof, and dimers of the analogues and derivatives which exhibit substantially no cross-reactivity with human C5a. The antibodies are used to detect or quantify circulating C5a analogue or derivative, as well as to modify, e.g., neutralize, the activity of the C5a receptor antagonist *in vivo*.



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C5a RECEPTOR ANTAGONISTS HAVING SUBSTANTIALLY NO AGONIST ACTIVITY AND METHODS FOR PREPARATION

Field of the Invention

The present invention relates to the field of immunology, and more specifically to the treatment of complement-mediated diseases and inflammatory conditions in mammals.

Background of the Invention

Inflammation is a localized, protective event, elicited by injury, which serves to destroy, dilute or wall off both injurious agents and the injured tissues. It involves a complex series of events, including dilation of arteries, capillaries and venules, with increased vascular permeability, increased blood flow, and exudation of fluids and plasma proteins. These processes are often rapidly followed by adhesion of leukocytes to the vascular endothelium, with subsequent influx of the cells into the surrounding tissue.

The complement system, a major immunological defense mechanism against foreign substances, has been shown to influence each of the factors that comprise the inflammatory response. In general, complement comprises a set of proteins that work to eliminate microorganisms and other antigens from tissues and blood. This task is achieved either by complement components alone or in cooperation with antibodies or with cells that express complement receptors. More specifically, the system consists of about 30 plasma proteins, their corresponding cellular receptors and several membrane regulatory proteins. Kinoshita, Immunology Today, *12*:291-300 (1991). Activation of the complement system by, for example, antigen-antibody complexes or bacterial surface structures, triggers an amplification cascade of proteolytic cleavage and protein assembly events of the complement components, which ultimately leads to the destruction and final elimination of the foreign body. Muller-Eberhard, Annu. Rev. Biochem. *57*:321-347 (1988).

Several biologically active peptides are generated by the activation of the complement system. C5a, a glycoprotein containing 74 amino acids and having an M_r of about 11,000, is generated by the proteolytic cleavage of the N-terminal end of C5, the fifth component of complement, by C5 convertase. Nilsson et al., J. Immunol. *114*:815-822 (1975). The biological properties of C5a extend across a multitude of cells and tissues involved in both acute and chronic inflammatory processes. Hugli, CRC Crit. Rev. Immunol., *1*:321-366 (1981). Many of these properties are immunologically beneficial. C5a has been found to

mediate host defense mechanisms in response to various pathological conditions. C5a participates in a wide variety of specific biologic functions commonly associated with the inflammatory response, such as smooth muscle contraction, an increase in vascular permeability, wheal and flare generation when injected into human skin, histamine release from mast cells, and induction of the oxidative burst and lysosomal enzyme release from polymorphonuclear leukocytes (PMNLs). C5a stimulates measurable responses from every circulating white blood cell including basophils, eosinophils, monocytes, and neutrophils. Hugli, supra.; Bautsch et al., Immunobiol. *185*:41-52 (1992). C5a has further been found to be a potent chemoattractant. Fernandez et al., J. Immunol. *120*:109-115 (1978). This protein is a pivotal stimulus to the attraction of PMNLs such as phagocytic cells to the site of inflammation.

Complement is beneficial when directed against an appropriate target such as invading microorganisms or tumor cells, but has clear pathogenic potential if activated inappropriately. For instance, the anaphylatoxins, e.g., C5a, have been implicated as causative or aggravating factors in the pathogenesis of several inflammatory diseases such as adult respiratory distress syndrome and rheumatoid arthritis. Bautsch et al., Biochem. J. *288*:261-266 (1992); Haslett et al., J. Immunol. *142*:3510-3517 (1989). In particular, the aberrant presence of C5a in tissue has been detected in patients afflicted with rheumatoid arthritis, osteoarthritis, psoriasis and noncardiac pulmonary edema. Hammerschmidt, J. Amer. Med. Soc. *244*:199- (1980). C5a has been found to be a principal inflammatory mediator produced by complement activation by virtue of additional activities that include recruitment and stimulation of inflammatory leukocytes and augmentation of antibody production. See Mollison et al., Proc. Natl. Acad. Sci. USA *86*:292-296 (1989).

The *in vivo* or pharmacologic control of inflammation is presumed to be dependent on the modulation of chemotaxis. Three levels at which inhibition can occur have been recognized. These are (1) suppression of the leukocytic response to chemotactic stimuli; (2) prevention of chemotaxin generation; and (3) inactivation of the chemotaxins. In addition, because C5a exerts its various functions by binding to a specific C5a receptor found in the membrane of several human cells such as neutrophils, eosinophils and monocyte-derived cells, the inhibition of C5a-mediated chemotaxis, and in particular, the design of C5a receptor antagonists have attracted considerable attention.

U.S. 4,772,584 to Cleary et al. discloses polypeptides isolated from group A streptococci which inhibit the binding of C5a to PMNLs by cleaving a six amino acid peptide from the C-terminus of C5a. U.S. 4.692.511 to Hahn teaches polypeptide receptor

antagonists to C5a which contain an essential core tetrapeptide Tyr-Asp-Gly-Ala (SEQ ID NO. 1) or Asp-Gly-Ala-Tyr (SEQ ID NO. 2), or core tripeptide Asp-Gly-Ala which display C5a blocking activity.

U.S. 5,190,922, WO 90/09162 and 92/11858 to Abbott Laboratories disclose various oligopeptides which bind to C5a receptors and purportedly modulate anaphylatoxin activity. However, several of these molecules have been shown to retain significant agonist activity. See Mollison et al., "C5a Structural Requirements for Neutrophil Receptor Interaction," in *Progress in Inflammation Research and Therapy,* Birkhauser Verlag, Basel (1991) at pages 17-21; Kawai et al., J. Med. Chem. *35*:220-223 (1992); Kawai et al., J. Med. Chem. *34*:2068-2071 (1991); and Or et al., J. Med. Chem. *35*:402-406 (1992).

There remains a strong need for a potent and therapeutically effective C5a receptor antagonist which is substantially void of agonist activity. Further, there remains a strong need for an effective method for preparation of such a C5a receptor antagonist.

Summary Of The Invention

One aspect of the present invention is directed to polypeptidic analogues or polypeptide derivatives of human C5a which are C5a receptor antagonists and exhibit substantially no anaphylatoxin or agonist activity, and to dimeric forms of the analogues or derivatives.

Another aspect of the present invention is directed to fusion proteins comprising said polypeptidic analogues or polypeptide derivatives.

DNA molecules encoding the polypeptides (i.e. the analogues and derivatives thereof) or fusion proteins, plasmids, vectors and host cells transformed with the DNA molecules, and methods of preparing the C5a analogues, derivatives or fusion proteins are also provided.

Pharmaceutical formulations containing a C5a analogue, derivative or dimer thereof are advantageously used in methods for the treatment of C5a-mediated inflammatory conditions and diseases in mammals, and as a prophylactic to prevent such inflammation.

Another aspect of the present invention is directed to antibodies specific to the C5a analogue or polypeptide derivative, which exhibit substantially no cross-reactivity with human C5a. The antibodies are used to detect or quantify circulating C5a analogues or derivatives in subjects (previously administered with same) as well as to modify, e.g., neutralize, the activity of the C5a analogues and derivatives *in vivo*.

Brief Description Of The Figures

Fig. 1 is a flow diagram that illustrates the synthesis of a synthetic gene encoding human C5a via oligonucleotide coupling; and

Fig. 2 is a plasmid map of pB-6/C5a.

Description Of The Preferred Embodiments

The C5a polypeptide analogues or polypeptide derivatives of human C5a of the present invention are C5a receptor antagonists which have substantially no agonist activity. The term "C5a receptor" is understood in the art as referring to the sites on the surfaces of human blood cells such as PMNLs and monocytic cells, to which C5a, its degradation product C5a-desArg, and the instant antagonists bind. See, for example, U.S. 5,177,190; and Oppermann et al, J. Immunol. *151(7)*:3785-3794 (1993). C5a is converted enzymatically to C5a-desArg in human serum by a carboxypeptidase B-like enzyme, and is the major physiological end product in man. Chenoweth et al., Mol. Immunol. *17*:151-161 (1980).

By the term "antagonist," it is meant that the instantly disclosed polypeptides are inhibitors of C5a. That is, they interfere with the binding of C5a to the C5a receptor. While not intending to be bound by any particular theory, Applicants believe that the C5a analogues or polypeptide derivatives of human C5a are competitive inhibitors of C5a in that they compete with C5a for binding to the C5a receptor.

The antagonism of the instant C5a analogues or polypeptide derivatives of human C5a may be quantified as an IC_{50} in the calcium rise assay disclosed in *Seligmann et al.*, Agents and Actions *21*:375-378 (1987), described in detail in Example 7. The IC_{50} is defined as the concentration of C5a analogue or of the polypeptide derivative of human C5a which inhibits 50% of the intracellular mobilization of calcium ions by the PMNLs bearing the C5a receptor, after a challenge dose with 100 pM human C5a. The C5a receptor antagonists of the present invention exhibit an IC_{50} of no greater than about 2.0×10^{-6} M in the calcium rise assay disclosed in *Seligmann et al.*, supra.

By the phrases "substantially no anaphylatoxin activity" or "substantially no agonist activity," it is meant that the binding of the C5a analogue or of the polypeptide derivative of human C5a (hereinafter used interchangeably with C5a receptor antagonist) to the receptor does not result in an endogenous signal transduction event ultimately resulting in the physiological responses commonly associated with anaphylatoxin-induced inflammation caused by binding of C5a to its receptor, such as activation of phagocytic cells, smooth muscle contraction, increase in vascular permeability, and excessive production of

inflammatory mediators, e.g., histamines, prostaglandins, thromboxanes, leukotrienes, interleukin (IL)-1, IL-6 and IL-8. See Hugli et al., CRC Crit. Rev. Immunol. *1*:321-326 (1981) and PCT WO 92/10205. A quantitative measure of this property may also be obtained using the calcium rise assay disclosed in *Seligmann et al., supra*, also described in Example 7. EC_{50} is a measure of agonistic activity. For purposes of the present invention, the EC_{50} value is that concentration of C5a analogue or of the polypeptide derivative of human C5a which produces 50% of the maximum response caused by that same C5a analogue or polypeptide derivative of human C5a, respectively. Applicants have not detected agonist activity of the instant C5a analogues or polypeptide derivatives of human C5a up to a concentration of at least about 8.0×10^{-7} M, and preferably at least about 3.0×10^{-6} M in the same calcium rise assay. The C5a analogues or the polypeptide derivatives of human C5a of the present invention are those for which the EC_{50} is not measurable in the Seligmann calcium rise assay up to C5a analogue or polypeptide derivative concentrations of at least about 8.0×10^{-7} M, and preferably at least about 3.0×10^{-6} M, since no response can be detected in the assay.

C5a is a 74-amino acid polypeptide, the sequence of which has been disclosed in Fernandez et al., J. Biol. Chem. *253*:6955-6964 (1978). Synthetic genes, constructed based upon the deduced nucleotide sequences, are disclosed in Mandecki et al, Proc. Nat'l Acad. Sci. USA *82*:3543-3547 (1985) and U.S. 4,937,189 to Davidow et al. The amino acid sequence of C5a disclosed in Fernandez, and the corresponding synthetic nucleotide sequence disclosed in Davidow et al. are set forth in Table 1, below.

Table 1

```
                 1   2   3   4   5   6   7   8   9   10
EcoRI           thr leu gln lys lys ile glu glu ile ala
                (SEQ. ID. NO.3)
AATTCT  ATG  ACT CTG CAA AAG AAG ATC GAA GAA ATC GCT
    GA  TAC  TGA GAC GTT TTC TTC TAG CTT CTT TAG CGA
    (SEQ. ID. NO. 4)

  11  12  13  14  15  16  17  18  19  20  21  22  23
 ala lys tyr lys his ser val val lys lys cys cys tyr

 GCT AAG TAC AAG CAC TCC GTC GTT AAG AAG TGT TGT TAC
 CGA TTC ATG TTC GTG AGG CAG CAA TTC TTC ACA ACA ATG
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 24  25  26  27  28  29  30  31  32  33  34  35  36
asp gly ala cys val asn asn asp glu thr cys glu gln

GAT GGT GCA TGC GTC AAC AAC GAC GAA ACC TGT GAA CAA
CTA CCA CGT ACG CAG TTG TTG CTG CTT TGG ACA CTT GTT


 37  38  39  40  41  42  43  44  45  46  47  48  49
arg ala ala arg ile ser leu gly pro arg cys ile lys

CGA GCT GCT CGT ATT TCT CTG GGC CCT CGC TGT ATC AAG
GCT CGA CGA GCA TAA AGA GAC CCG GGA GCG ACA TAG TTC

 50  51  51  53  54  55  56  57  58  59  60  61  62
ala phe thr glu cys cys val val ala ser gln leu arg

GCT TTC ACT GAA TGT TGT GTT GTC GCT TCC CAA CTG CGC
CGA AAG TGA CTT ACA ACA CAA CAG CGA AGG GTT GAC CCG


 63  64  65  66  67  68  69  70  71  72  73  74
ala asn ile ser his lys asp met gln leu gly arg stop
                                                HindIII
GCT AAC ATT TCT CAC AAG GAC ATG CAA CTC GGC CGC TAA A
CGA TTG TAA AGA GTG TTC CTG TAC GTT GAG CCG GCG ATT TTCGA
```

Applicants have unexpectedly and surprisingly discovered that certain analogues or polypeptide derivatives of human C5a, produced by mutagenizing the portion of a synthetic C5a gene encoding the C-terminal region, i.e. amino acids 64-74, of human C5a (hereinafter used interchangeably with "C5a(1-74)"), have dramatically different properties than C5a. That is, they exhibit excellent antagonistic properties and substantially no agonist activity. The N-terminal amino acid residue of such analogues or polypeptide derivatives of human C5a may be any amino acid residue, for example a Thr residue as in human C5a, or a Met residue. In particular, said polypeptide derivative of human C5a has a glycine residue as the N-terminus.

Thus, in one embodiment of the present application there is provided a polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, said derivative having a glycine residue as the N-terminus.

Preferably the N-terminal glycine residue can be in the form of an adduct (N-terminal additional residue), or, also preferred, said glycine residue is a substituent for the N-terminal amino acid threonine of human C5a (substitution Thr1Gly).

In particular, polypeptide derivatives of human C5a according to the present application can be produced for example by mutagenizing the N-terminal amino acid residue of human C5a threonine to glycine, and further mutagenizing the portion of a synthetic C5a gene encoding the C-terminal region, i.e., amino acids 64-74, of human C5a (hereinafter used interchangeably with "C5a(1-74)").

Specifically, the C5a analogues or polypeptide derivatives of human C5a of the present invention are defined in terms of at least one or, preferred, both of two modifications or mutations to the C-terminal region

```
of C5a (1-74), N'-Asn-Ile-Ser-His-Lys-Asp-Met-Gln-Leu-
                    (64)(65)(66)(67)(68)(69)(70)(71)(72)
Gly-Arg-C' (SEQ ID NO. 5),
(73)(74)
```

(amino acids 64-74 of C5a (1-74)). First, it is truncated at least to Leu (72); i.e., by removing the Gly (73) and Arg (74) residues. Second, at least one cysteine is substituted in the region, provided, in a most preferred embodiment, that the C-terminal amino acid of the polypeptide (i.e., the C-terminus) is cysteine, and that the thiol (SH) group of the C-terminal cysteine is in reduced form (i.e., has a free thiol group), or is in a form capable of spontaneously converting or being readily converted into a free thiol group. In another preferred embodiment the cysteine residue is in the form of an adduct (C-terminal additional residue).

Thus, in a specific embodiment such a polypeptide derivative comprises a C-terminal region which differs from the corresponding C-terminal region of human C5a, in that it has a cysteine residue, and is truncated at its C-terminus by at least two amino acid residues.

In a preferred embodiment, from 2 to 6 of the most C-terminal amino acids are truncated from C5a (1-74). Accordingly, a preferred polypeptide derivative of the present invention is from 64 to 72 amino acids, preferably from 68 to 72 amino acids, more preferred from 70 to 72 amino acids, and most preferred 71 amino acids in length.

Thus, in the case where the N-terminal 63 amino acid region is kept intact and only one cysteine is substituted, the respective corresponding examples or embodiments may be designated for example as follows: C5a (1-72, Leu72Cys), C5a (1-72, Thr1Met, Leu72Cys), preferred C5a (1-72, Thr1Gly, Leu72Cys); C5a (1-71, Gln71Cys), C5a (1-71, Thr1Met, Gln71Cys), preferred C5a (1-71, Thr1Gly, Gln71Cys); C5a (1-70, Met70Cys), C5a (1-70, Thr1Met, Met70Cys), preferred C5a (1-70, Thr1Gly, Met70Cys); C5a (1-69, Asp69Cys), C5a (1-69, Thr1Met, Asp69Cys), preferred C5a (1-69, Thr1Gly, Asp69Cys); and C5a (1-68, Lys68Cys), C5a (1-68, Thr1Met, Lys68Cys), preferred C5a (1-68, Thr1Gly, Lys68Cys). In a more preferred embodiment, the C-terminal region is truncated to and including Met70, Gln71 or Leu72, which correspond to the former designated embodiments.

The C-terminal region can be further truncated N-terminally to Asn 64, which would correspond to the representative designated examples or embodiments C5a (1-67, His67Cys), C5a (1-67, Thr1Met, His67Cys), preferred C5a (1-67, Thr1Gly, His67Cys); C5a (1-66, Ser66Cys), C5a (1-66, Thr1Met, Ser66Cys), preferred C5a (1-66, Thr1Gly, Ser66Cys); C5a (1-65, Ile65Cys), C5a (1-65, Thr1Met, Ile65Cys), preferred C5a (1-65, Thr1Gly, Ile65Cys); and C5a (1-64, Asn64Cys), C5a (1-64, Thr1Met, Asn64Cys), preferred C5a (1-64, Thr1Gly, Asn64Cys); provided that the resultant C5a analogue or polypeptide derivative of human C5a exhibits the forementioned requisite antagonist property (an IC_{50} of no greater than about 2.0×10^{-6} M) and substantially no anaphylatoxin or agonist activity (a non-measurable EC_{50} up to C5a analogue or polypeptide derivative concentrations of at least 3.0×10^{-6} M). Those skilled in the art would understand that "analogues" or "polypeptide derivatives" of human C5a do not include antibodies specific to C5a or to sites on the C5a receptor.

Further derivatives of human C5a as described herein are included within the scope of the present invention. These include modifications such as point mutations, substitutions, additions and deletions in the N-terminal 63 amino acid region (amino acids 1-63 of C5a(1-74)), *Carney et al.*, Protein Science *2*:1391-1399 (1993), and further amino acid substitutions in the thus-mutagenized C-terminal region. The type and extent of the modifications are generally not important, so long as the resultant derivative remains a C5a receptor antagonist with substantially no agonist activity, both as defined above. For example, the Cys27 residue in the N-terminal region of C5a (1-74) can be changed, e.g., to a serine residue, in order to minimize complications during refolding. Thus, the C5a analogue derivative is designated for example C5a(1-71, Cys27Ser, Gln71Cys), or C5a(1-71, Thr1Met, Cys27Ser, Gln71Cys), or in a preferred embodiment C5a(1-71, Thr1Gly,

Cys27Ser, Gln71Cys). Also, the N-terminus may be changed to a Methionine residue, either by substitution or addition, to allow for expression of a C5a analogue or polypeptide derivative in various host cells such as E. coli. Any C5a analogue mentioned herein directly produced in E. coli will have a Met residue as its N-terminus, as explained in Example 1 and shown in Table 3 (Example 3, below). Further, C5a analogues or polypeptide derivatives having a Met residue as the N-terminus may be produced in the form of a fusion protein, with subsequent cleavage of the fusion part.

N-terminal substitutions also occur in cases where the human C5a analogues or polypeptide derivatives of the present invention are expressed in various host cells, e.g., E. coli, as a fusion protein, and then isolated by cleaving the fusion protein at a convenient site. For example, cleavage of the polypeptide derivative of human C5a from its fusion protein partner linked via an hydroxylamine linkage, results in the substitution of a glycine (Gly) residue for the native human C5a threonine (Thr) N-terminus. Preferred embodiments of polypeptide derivatives having the substitution C5a(Th1Gly) have been mentioned above. Of these, the most preferred embodiments are C5a (1-71, Thr1Gly, Gln71Cys) or C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys).

An example of a further modification of the C-terminal region is the substitution of a Phenylalanine residue for the native Histidine at position 67 of C5a(1-74). As an example, a C5a analogue derivative is designated C5a(1-71, Thr1Met, Cys27Ser, His67Phe, Gln71Cys). Thus, in another preferred embodiment, the polypeptide derivatives of human C5a are those mentioned above, comprising the substitution C5a(His67Phe). Preferred is the polypeptide derivative of human C5a designated C5a (1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys).

The C5a analogues or polypeptide derivatives of human C5a of the present invention can be prepared via numerous procedures standard in the art. For instance, they may be prepared via direct chemical synthesis. They may also be prepared by expression of DNA molecules, i.e., synthetic genes, encoding the polypeptides in suitable host cells.

Thus, according to a further embodiment of the present application, there is provided a DNA molecule encoding a polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, said derivative having a glycine residue as the N-terminus. Preferred embodiments thereof are DNA molecules encoding the polypeptide derivatives of human C5a of the present invention as mentioned herein. Of these, preferred are DNA molecules encoding polypeptide derivatives of human C5a of the present invention, which are 64 to 72, preferably 71, amino acid

residues in length. Even more preferred are DNA molecules encoding the polypeptide derivative designated C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys) or C5a (1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys).

These DNA molecules, deducible from the amino acid sequences of the analogues or polypeptide derivatives of human C5a, in turn may be prepared via known techniques. The DNAs may be synthesized chemically as disclosed in Narang, Tetrahedron *39*:3-22(1983) and EPA 146,785; Mandecki et al., Proc. Natl. Acad. Sci. USA *82*:3543-3547 (1985) (disclosing the chemical synthesis of a gene encoding C5a). Fragments of the DNA molecules may be prepared chemically, which then are linked together enzymatically. See Volume 1, Chapter 8 of *Current Protocols in Molecular Biology*, Ausubel et al. (Eds.), Wiley, NY (1990).

DNAs encoding C5a analogues or polypeptide derivatives of human C5a of the present invention can also be prepared by mutagenesis of known synthetic or natural genes encoding C5a, such as those disclosed in Fernandez, Mandecki and Davidson, for example. See Ausubel et al., *supra.;* Volume II, Chapter 15 of Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor, NY (1989); and Mollison et al., Proc. Natl. Acad. Sci. USA *86*:292-296 (1989). Further, the DNAs may be prepared via PCR techniques. *PCR Protocols*, Innis et al. (Eds.), Academic Press, San Diego, CA (1990).

The DNA molecules encoding the C5a analogues or polypeptide derivatives of the present invention are operably linked to known regulatory sequences, e.g., promoter, enhancer, 3'-untranslated sequences, and 5' translated sequences, e.g., signal and leader sequences, and then transformed into host cells capable of expressing the genes, in accordance with art-recognized techniques. Then, the transformed host cells are cultured under conditions suitable for expression of the antagonist encoding gene. Representative host cells include prokaryotes such as *E. coli* and *Bacillus*, e.g., *B. subtilis*; and eukaryotes such as filmentous fungi, e.g., *Aspergillus niger*,, yeast, e.g., *Saccharomyces cerevisiae*, *Pichia pastoris* and *Yarrowia lipolytica*; baculovirus/insect cell cultures (Summers et al., *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station (1987)); mammalian cell lines; and plants (J. Vandekerckhove et al., BIO/TECHNOLOGY 7:929-932 (1989)).

In general, the procedures for expression of C5a in *E. coli* are applicable to gene expression of C5a analogue or of polypeptide derivative of human C5a. See, Mandecki, Proc. Natl. Acad. Sci. USA *82*:3543-3547 (1985); Mollison et al., Proc. Natl. Acad. Sci. USA

86:292-296 (1989); and Bautsch et al., Immunobiol. *185*:41-52 (1992). The choice of suitable regulatory sequences such as promoter (e.g., T7 polymerase, UV5-D, trp or lac), ribosome binding site, as well as suitable plasmid vectors containing transcriptional stop sites, e.g., pKK223-2, are within the level of skill in the art. To optimize expression in *E. coli*, the DNA molecule should be synthesized using *E. coli*-preferred codons as disclosed in Guoy et al., Nucleic Acids Res. *10*:7055-7074 (1982), and to allow for several restriction endonuclease sites to facilitate characterization of the synthesized DNA and possibly mutagenesis of the DNA sequence. This approach allows for direct expression of the C5a analogue or polypeptide derivative of the present invention by introducing an ATG initiation codon for protein synthesis directly and immediately upstream of the triplet coding for the first amino acid of the polypeptide. Further, *E. coli* strains, e.g., *lon*, which are deficient in one of several proteases present in wild-type cells offer the advantage of increased yield of protein. Franke et al., Meth. Enzymol. *162*:653-658 (1988).

Thus, a further embodiment of the present application is directed to a recombinant DNA-molecule, comprising a promoter capable of functioning in a given host operably linked to a DNA molecule encoding a C5a anlogue or a polypeptide derivative of human C5a of the present invention. Furthermore, the present application is directed to a recombinant plasmid compatible with a given host, comprising a recombinant DNA molecule as mentioned above encoding a C5a analogue or a polypeptide derivative of human C5a of the present invention. In a broader sense an embodiment is directed to a recombinant vector compatible with a given host, comprising a recombinant DNA molecule as emntiojed above encoding a C5a anlogue or a polypeptide derivative of human C5a of the present invention.

Expression of the C5a analogue genes in hosts such as *E. coli* may also be enhanced by expressing the genes in the form of a fusion protein. Such a fusion protein being a further embodiment of the present invention, generally consists of, in the order of N-terminus to C-terminus, a fusion partner, a cleavable linker, and, fused thereto, a polypeptide derivative of human C5a, wherein said polypeptide derivative is a C5a receptor antagonist that exhibits substantially no agonist activity. Within the scope of the present application, a polypeptide derivative of human C5a forming part of such a fusion protein is not restricted to those particular analogues or polypeptide derivatives of the present invention as mentioned above, which, however, form a preferred embodiment. Rather, such an analogue or derivative can be any other C5a analogue or polypeptide derivative of

human C5a, as long as such an analogue or derivative is a C5a receptor antagonist derived from human C5a that exhibits substantially no agonist activity. In particular, further preferred analogues or derivatives forming part of said fusion protein are derived by introducing any N-terminal substitution of human C5a into the analogues or derivatives of the present invention, e.g. the substitution Thr1Gly or Thr1Met. Likewise, an analogue or polypeptide derivative of human C5a without a substitution at the N-terminus of human C5a (Thr1) can be used in the fusion protein. In the alternative, there can be added any other amino acid to the N-terminus of said analogue or polypeptide derivative of human C5a, giving an adduct. Preferred is a fusion protein wherein such an analogue or polypeptide derivative of human C5a further comprises a C-terminal region which differs from the corresponding C-terminal region of human C5a, in that it has a cysteine residue, and is truncated at its C-terminus by at least two amino acid residues, as described above. More preferred, the fusion protein comprises such an analogue or polypetide derivative of human C5a having a cysteine residue as the C-terminus, either as an adduct, or, more preferred, as a substituent.

Methods of preparing such fusion proteins using chemically and enzymatically cleavage linkages are known in the art. See, e.g., Smith, in "Methods in Molecular Biology," Vol. 3, New Protein Techniques, pp. 57-70 and 71-88 (1984), and Van Heeke et al., Protein Expression and Purification 4:265-74 (1993). Fusion partners, in general, are those genes (or fragments thereof) which are highly expressed in the host, e.g., *E. coli*. In the case of *E. coli*, suitable fusion partners include endogenous *E. coli* genes and synthetic genes, e.g., containing *E. coli*-preferred codons. In addition, there may be used other fusion partners known in the art, for example human carbonic anhydrase II (see example 16 for details). In principle, the smaller the size of the fusion partner, the better the yield of the C5a analogue or polypeptide derivative of human C5a.

In a most preferred embodiment of the present application a fusion protein, consisting of, in the order of N-terminus to C-terminus, a fusion partner, a cleavable linker and, fused thereto, a polypeptide derivative of human C5a, wherein said polypeptide derivative is a C5a recepto- antagonist that exhibits substantially no agonist activity, wherein said fusion partner is a polypeptide capable of directing the formation of inclusion bodies in a cell, preferably in an *E.coli* cell, is provided.

Applicants have unexpectedly and surprisingly found that it is possible to direct the formation of inclusion bodies, in particular in *E. coli*, with a fusion protein according to the

present invention, in particular wherein said fusion partner together with said cleavable linker is a polypeptide about the amino acid length (or molecular weight) or even less of said polypeptide derivative of human C5a. Thus, a specific embodiment of the present invention is directed to a fusion protein as defined above, wherein said fusion partner is a polypeptide capable of directing the formation of inclusion bodies in a cell, in particular an E.coli cell. Preferred is a fusion protein wherein said fusion partner together with said cleavable linker is a polypeptide about the amino acid length (or molecular weight) or even less of said polypeptide derivative of human C5a. Thus, where, for example, a preferred polypeptide derivative of human C5a as mentioned above has a length of 64 to 72 amino acids, said fusion partner together with said linker preferably has about the same length, preferably less than said length, preferred 42 to 61 amino acids, or even less. Said fusion partner itself preferably has a length of 40 to 53 amino acids, or even less. Preferably, the fusion partner comprises an N-terminal fragment of human interleukin-1β (also designated as IL-1β or IL-1B) or a mutant therof. As will be appreciated by those of skill point mutations, e.g. the single point mutation like human IL-1β (Cys9Ser), can be performed on the N-terminal fragment of human IL-1β, resulting in a further preferred fusion partner capable of directing the formation of inclusion bodies. For example, said fusion partner comprises amino acid residues 1 to 47 of human IL-1β (Dinarello, Blood 77: 1627-52 (1991), or a mutant thereof as mentioned above. Optionally additionally said fusion partner comprises a short fragment of human interleukin 1 receptor antagonist (also designated as IL-1RA), for example having a length of 3 to 6 amino acids (Dinarello, supra) located C-terminally to said N-terminally fragment of human IL-1β. Those skilled in the art can determine the appropriate size of a fusion partner in accordance with standard techniques (e.g. by determining expression titers of C5a analogue fusion proteins by systematically varying the size (length) of the fusion partner).

In a preferred embodiment for expression in *E. coli*, the fusion partner is encoded by a 159-nucleotide DNA encoding a 53 amino acid fragment of the hybrid protein human interleukin 1 beta and interleukin 1 receptor antagonist, (Dinarello, Blood 77: 1627-52 (1991), which DNA contains *E. coli*-preferred codons. Such a fusion partner for example consists, in the order of N-terminus to C-terminus, of amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above and amino acid residues 52 to 57 of human interleukin 1 receptor antagonist (Dinarello, see above). Also preferred as fusion partner for expression in *E. coli* is a 50 amino acid fragment (encoded by a 150 nucleotide DNA

containing *E. coli*-preferred codons) consisting of a hybrid protein of human interleukin 1 beta and interleukin 1 receptor antagonist. Such a fusion partner for example consists of, in the order of N-terminus to C-terminus, amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above and amino acids 52 to 54 of human interleukin 1 receptor antagonist (Dinarello, see above).

In a preferred embodiment the cleavable linker of a fusion protein of the present invention comprises a hydroxylamine sensitive site (Asn-Gly) (also called hydroxylamine cleavage site) (e.g. Carrey,E., in Creighton,T.E. (ed): Protein Structure, A Practical Approach, IRL Press, Oxford, UK (1989)). Optionally additionally, such a linker may comprise an enterokinase protease cleavage site of the amino acid sequence -Val-Asp-Asp-Asp-Asp-Lys- (SEQ.ID. No. 68), kown in the art (e.g. Van Heeke et al., Protein Expression and Purification 4: 265-274 (1993)), located N-terminally to said hydroxylamine cleavage site, said linker being encoded by a 24 nucleotide DNA fragment. For expression in E. coli such linkers are encoded by DNA molecules containing E. coli preferred codons. As a skilled person is aware of, the linker comprising the cleavage site(s) mentioned above may comprise additional amino acid residue(s) in order to facilitate cleavage, as long as the overall lenght (or molecular weight) of said fusion partner together with said linker is about the length (or molecular weight) or less of the polypeptide derivative of human C5a to be produced. For example there may be present additional 1, 2 or 3 or even more amino acid residue(s) located between the fusion partner and any of said cleavage site(s). In the alternative, if more than one cleavage site is present, the additional amino acid residue(s) may be present between each two of said cleavage sites.

Thus, in a preferred embodiment a fusion protein according to the present invention consists of, in the order of N-terminus to C-terminus, amino acid residues 1 to 47 of human IL-1β or a mutant thereof as emntioned above, and amino acid residues 52 to 54 of human IL-1RA, a cleavable linker sequence comprising the amino acid residues -Val-Asp-Asp-Asp-Asp-Lys-Asn-Gly- (SEQ. ID. NO. 69), and a polypeptide derivative of human C5a as defined above, wherin the C-terminal amnio acid residue Gly of said cleavable linker is a substituent for the N-terminal amino acid residue Thr of human C5a in said polypeptide derivative. In this context, preferred polypeptide derivatives are those as described above, for example the polypeptide derivative of the present invention being from 64 to 72, preferably 71, amino acid residues in length. Preferred examples of such derivatives are C5a(1-71, Thr1Gly, Cys27Ser, Gln71Cys) or C5a(1-71, Thr1Gly, Cys27Ser, Hus67Phe, Gln71Cys).

Further embodiments of the present invention are directed to a DNA molecule encoding a fusion protein as mentioned above as prefered embodiments. For example, preferred is a DNA molecule encoding a fusion protein as mentioned above, wherein said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, and amino acid residues 52 to 57 of human IL-1RA, or wherein said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, and amino acid residues 52 to 54 of human IL-1RA. A particular preferred example is a DNA molecle encoding a fusion protein , consisting of, in the order of N-terminus to C-terminus, amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, amino acid residues 52 to 54 of human IL-1RA, a cleavable linker comprising the amino acid sequence -Val-Asp-Asp-Asp-Asp-Lys-Asn-Gly-, and a polypeptide derivative as defined in claim 1, wherein the C-terminal residue Gly of said linker is a substituent for the N-terminal amino acid residue Thr of human C5a in said polypeptide derivative.

Another embodiment of the present invention is directed to a recombinant DNA molecule, comprising a promoter capable of fuctioning in a given host operably linked to a DNA molecule encoding a fusion protein according to the present invention as defined above. Another embodiment is directed to a recombinant plasmid or, in a broader sense, to a recombinant vector, respectively, compatible with a given host, comprising a recombinant DNA molecule as defined above. Preferred embodiments of said recombinant DNA molecule, plasmid or vector, respectively, are those as described above in connection with said DNA molecules and/or said fusion proteins.

Methods of preparing said DNA molecules, recombinant DNA, plasmid or vector, respectively, are similar to those methods as described above.

In general, the C5a analogue-encoding or polypeptide derivative-encoding synthetic genes can be expressed in yeast by following known procedures. See, for example, Romanos et al., Yeast *8*:423-488 (1992); Section IV of Goeddel (Ed.), Meth. Enzymol. *185*:231-484 (1990); Davidow et al., *supra.* and U.S. 4,775,622. To optimize expression in yeast, the DNA molecule should be prepared using yeast-preferred codons, particularly to avoid Arg-Arg pairs which are targets for endogenous KEX2 proteases. The use of glutamine, as opposed to methionine, as the N-terminus, facilitates proteolytic cleavage from the signal sequence, e.g., alpha factor signal sequence. It is further preferred to

eliminate any potential glycosylation sites such as the Asparagine at position 64 of various embodiments of the instant C5a receptor antagonists.

Expression of a C5a analogue- or polypeptide derivative-encoding gene of the present invention in mammalian cells can be performed in accordance with known procedures. See Chapter 16, "Expression of Cloned Genes in Mammalian Cells," in Maniatis et al., supra. A representative method of expression in human cells is disclosed in Berg et al., BioTechniques *14(6)*:972-978 (1993). Suitable human cells include publicly available cell lines such as HeLA S3 (ATCC CCL2.2) and HEK293 (ATCC CRL1573). Expression in CHO cells is disclosed, for example, in Asselbergs et al., Fibrinolysis *7*:1-14 (1993). Suitable hamster cell lines include CHO-K1 (ATCC CCL61), BHK (ATCC CRL6281), BHK-21 (ATCC 6281, CCL10 and CRL8544). Representative monkey cells are CV-1 (ATCC CCL70), COS-7 (ATCC CRL1650), and VERO cells (ATCC CCL81). A suitable mouse cell line is C127 (ATCC 1804). Preferred cell lines are DHFR-minus CHO lines as disclosed in Uriaub et al., Proc. Natl. Acad. Sci. USA *77*:4216-4220 (1980). Serum-independent cell lines are more preferred. See Kurano et al., Bio/Technology *16*:245-258 (1990). In mammalian hosts, glycosylated or non-glycosylated forms of the C5a analogues can be produced.

Thus, a further embodiment of the present invention is directed to a recombinant host, stably transformed with a recombinant DNA molecule, comprising a DNA molecule encoding a C5a analogue or polypeptide derivative of human C5a, or encoding a fusion protein, all as defined above. Preferably , said recombinant host is selected from the group consisting of bacterial, fungal, insect, mammalian and plant cells, a skilled person will know of. Particularly preferred in either case as recombinant host is E. coli.

The C5a analogues or polypeptide derivatives of human C5a isolated from transformed *E. coli* cells are renatured to assume biological activity wherein the C-terminal cysteine is in reduced form, i.e., it contains a free thiol group, preferably by using a convenient one-step procedure. Applicants have unexpectedly discovered that treating the denatured C5a analogue with a redox couple in a molar ratio of reducing agent to oxidizing agent from at least about 100:1 to about 500:1 results in a biologically active C5a analogue having a C-terminal cysteine in reduced form. This ratio is from about 10-fold to about 50-fold greater than known ratios (a preferred ratio of reduced sulfhydryl to oxidized sulfhydryl compound of 10:1 is disclosed on col. 17, lines 43-45 of Builder et al., U.S. 4,620,948). In accordance with the procedure, the transformed *E. coli* cells, after culturing under conditions sufficient to cause production of the C5a analogue, are mixed with a denaturing

and solubilizing agent, e.g., 6M guanidine HC1, to produce denatured C5a analogue, optionally with further disruption by any known technique such as sonication, French Press or DynoMill. The thus-mixed or thus-disrupted cells containing the denatured C5a analogue are then mixed with a redox couple in a molar ratio by weight of reducing agent/oxidizing agent of from at least about 100:1 to about 500:1 under suitable conditions to produce renatured, biologically active C5a analogue. Thus, another preferred embodiment of the present invention is directed to a method of preparing a biologically active polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, said derivative having a glycine residue as the N-terminus, comprising the steps of:

(1) culturing *E. coli* cells stably transformed with a DNA molecule encoding said polypeptide derivative under conditions suitable to cause expression of the DNA molecule;

(2) contacting the thus-cultured cells with a denaturing and solubilizing agent to produce said polypeptide derivative in denatured form; and

(3) mixing the thus-denatured polypeptide derivative with a solution containing a reducing agent and an oxidizing agent in a molar ratio of the reducing agent to the oxidizing agent by weight of at least about 100:1 under suitable conditions to produce the polypeptide derivative in biologically active form.

Suitable redox couples include cysteine/cystine and reduced glutathione/oxidized glutathione. Others skilled in the art will appreciate that other redox couples can be used. The glutathione redox couple is preferred. Suitable conditions include a pH of from 6.5 to 7.5, preferably 7.4. The mixture is allowed to stand at room temperature for a time sufficient to maximize the yield of protein. The preferred time is from about 1/2 hour to about 4 hours. Thus, this method eliminates the need to isolate the refractile, inclusion bodies (i.e., the insoluble mass of expressed protein) from the bacterial cells, and then to reduce the thiol group of the C-terminal cysteine.

As it may be desirable to express the C5a analogue or the polypeptide derivative of human C5a as a fusion protein, a further embodiment of the present invention is directed to a method as stated above, wherein said DNA molecule encodes the polypeptide derivative in the form of a fusion protein. Preferably, such a method further comprises the step of cleaving the thus-expressed fusion protein prior to said step of mixing. Hence, there is provided a method of preparing a biologically active C5a analogue or polypeptide derivative of human C5a, wherein said analogue is a C5a receptor antagonist that exhibits substantially no agonist activity, comprising the steps of

(1) culturing host cells stably transformed with a recombinant DNA molecule encoding said polypeptide derivative C5a analogue in the form of a fusion protein wherein said culturing is conducted under conditions suitable to cause expression of said fusion protein;
(2) isolating the fusion protein from the thus-cultured host cells; and
(3) cleaving the thus-isolated fusion protein so that the C5a analogue or polypeptide derivative of human C5a can be obtained.

Preferred embodiments of fusion proteins and of polypeptide derivatives of human C5a comprised by such fusion proteins are those fusion proteins or polypetide derivatives, respectively, as described above. Preferred polypeptide derivatives are from 64 to 72 amino acid residues in length, and are for example C5a(1-71, Thr1Gly, Cys27Ser, Gln71Cys) or C5a(1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys). Further reaction conditions of the said method are those as described above.

An embodiment where the C5a analogue or polypeptide derivative thereof is expressed in the form of a fusion protein by way of inclusion bodies, is directed to a method of preparing a biologically active polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, comprising the steps of:
(1) culturing host cells stably transformed with a recombinant DNA molecule encoding said polypeptide derivative C5a analogue in the form of a fusion protein wherein said culturing is conducted under conditions suitable to cause expression of said fusion protein in the form of inclusion bodies;
(2) isolating the inclusion bodies containing the fusion protein from the thus-cultured host cells;
(3) contacting the thus-isolated inclusion bodies with a denaturing and solubilizing agent to produce the fusion protein in denatured form;
(4) cleaving the thus-isolated fusion protein so that the polypeptide derivative of human C5a can be obtained; and
(5) mixing the thus-cleaved fusion protein with a solution containing a reducing agent and an oxidizing agent in a molar ratio of the reducing agent to the oxidizing agent by weight of at least about 100:1 under suitable conditions to produce the polypeptide derivative in biologically active form;
wherein steps 3 and 4 are carried out simultaneously or consecutively.

In general, preferred embodiments of DNA molecules, fusion proteins, polypeptide derivatives and host cells are those as mentioned above. Thus, preferably said host cells

are E.coli cells. In a preferred embodiment said recombinant DNA molecule encodes a fusion protein consisting of, in the order of N-terminus to C-terminus, a fusion partner, a cleavable linker and, fused thereto, a polypeptide derivative of human C5a, wherein said polypeptide derivative is a C5a receptor antagonist that exhibits substantially no agonist activity. As mentioned above, said derivative preferably comprises a C-terminal region which differs from the corresponding C-terminal region of human C5a, in that it has a cysteine residue, and is truncated at its C-terminus by at least two amino acid residues. In particular, said derivative has a cysteine residue as the C-terminus. In a most preferred embodiment said derivative has a glycine residue as the N-terminus.

In order to allow for the method to be performed effectively, said fusion partner together with said cleavable linker preferably is a polypeptide about the amino acid length (or molecular weight) of said polypeptide derivative of human C5a. In particular, said fusion partner comprises an N-terminal fragment of human IL-1β, as mentioned above. For example, said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, and amino acid residues 52 to 57 of human IL-1RA, or said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, and amino acid residues 52 to 54 of human IL-1RA. in a preferred embodiment said linker comprises a hydroxylamine cleavage site. More specific, said linker comprises, in the order of N-terminus to C-terminus, an enterokinase protease cleavage site and a hydroxylamine cleavage site.

Hence, such a fusion protein preferably consists of, in the order of N-terminus to C-terminus, amino acid residues 1 to 47 of human IL-1β or a mutant thereof as mentioned above, amino acid residues 52 to 54 of human IL-1RA, a cleavable linker comprising the amino acid sequence -Val-Asp-Asp-Asp-Asp-Lys-Asn-Gly- (SEQ. ID. No. 69), and a polypeptide derivative as defined in claim 1, wherein the C-terminal residue Gly of said linker is a substituent for the N-terminal amino acid residue Thr of human C5a in said polypeptide derivative. The derivative may be from 64 to 72 amino acid residues in length or may have another preferred length as mentioned above. Examples for preferred polypeptide derivatives in this context are C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys) or C5a (1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys).

In particular, the resultant inclusion body containing fusion protein can be recovered quantitatively after cell lysis using low speed centrifugation (e.g., about 3,000 x g), thus providing a relatively highly enriched fraction which then can be appropriately solubilized.

This procedure offers the advantage of using a 20-fold decrease in volume per weight of cells recovered from the fermenter.

In a preferred embodiment thereof, said mixing is conducted as described above, in particular at a pH of about 6.5 to about 7.5. Preferably said mixing is conducted for a period of time from about 1/2 hour to about 4 hours. The redox couple preferrably is reduced glutathione/oxidized glutathione, as described above.

In the alternative, the C5a analogues or polypeptide derivatives of human C5a may be renatured according to standard refolding and purification schemes such as disclosed in Builder et al., U.S. 4,620,948, for example. Following these procedures, the C-terminal cysteine will be in the form of an adduct, e.g., cys-cys or cys-glutathione. Therefore, the adduct must be further reduced to yield the free thiol group. Applicants have discovered that adducts of the disclosed C5a analogues or polypeptide derivatives of human C5a also function as C5a receptor antagonists which exhibit substantially no agonist activity as defined herein, and thus are included within the scope of the present invention. However, the further reduction would be necessary to prepare the preferred embodiments if these standard renaturation techniques were used.

Following renaturation, the C5a analogues or polypeptide derivatives of human C5a may be purified to the extent desired. Representative purification schemes include ultrafiltration, diafiltration, gel electrophoresis, chromatographic processes such as ion exchange chromatography, size exclusion chromatography, HPLC, reverse phase HPLC, treatment with Sephadex, dialysis, affinity chromatography, etc. Those skilled in the art would appreciate that a combination of purification schemes can be used.

C5a analogues or polypeptide derivatives of human C5a of the present invention having a C-terminal cysteine residue can be oxidized to form dimers in accordance with standard techniques. Thus, a further embodiment is directed to a dimer, comprising first and second polypeptide derivative of human C5a, wherein each of said derivatives is a C5a receptor antagonist that exhibits substantially no agonist activity, and has a C-terminal cysteine residue, and wherein the cysteine residues of said first and second derivatives are linked together via a disulfide linkage, and wherein said first and second polypeptide derivative of human C5a may be the same or different, and further wherein at least one of said first and second polypeptide derivatives of human C5a is a polypeptide derivative of human C5a as defined above i.e. having a glycine residue as the N-terminus. Preferably, in such a dimer the C-terminal region of each of said first and said second derivatives differs from the corresponding C-terminal region of human C5a in that it is truncated by at least two

amino acid residues. in a preferred embodiment in such a dimer each of said first and second derivatives is the human C5a derivative C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys). To prepare the dimers, the thiol (-SH) groups of the C-terminal cysteines of the respective monomers (analogues) are oxidized to produce a disulfide linkage. Homodimers and heterodimers are embraced by the term "dimer".

Another embodiment of the present invention is directed to a polypeptide derivative of human C5a of the present invention or a dimer thereof as defined above for use in the therapeutic or prophylactic treatment of a mammal inclusive man. A further embodiment is directed to the use of a polypeptide derivative of human C5a of the present invention or a dimer thereof as defined above for the preparation of a pharmaceutical composition for treatment of a C5a mediated disease or inflammatory condition in a mammal inclusive man.

Another embodiment is directed to a pharmaceutical composition useful in the treatment of a C5a-mediated disease or inflammatory condition in a mammal inclusive man, comprising a therapeutically effective amount of a polypeptide derivative of human C5a or dimer thereof of the present invention as defined above, and optionally a pharmaceutically acceptable carrier. In this context, preferred polypeptide derivatives or dimers are those which are mentioned as preferred compounds in accordance with the present invention (see above). For example, a pharmaceutical composition comprises preferably the polypeptide derivative of human C5a, which is C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys), or a dimer thereof comprising said derivative.

A further embodiment is directed to a method of treating a C5a-mediated disease or inflammatory condition in a mammal, comprising the step of administering a pharmaceutical composition as mentioned above to a mammal inclusive man in need thereof. Yet a further embodiment is directed to a method of reducing C5a-mediated inflammation in a mammal inclusive man, comprising the step of administering a pharmaceutical composition as mentioned above to said mammal at a time relative to a complement activation-causing or aggravating event sufficient to reduce the inflammation.

In particular, the C5a analogues or polypeptide derivatives of human C5a and dimers thereof of the present invention are useful in the treatment and/or prevention of injurious conditions or diseases in which the complement system, and more particularly C5a and analphylatoxin, are involved. They are therapeutically most effective when administered to any mammalian patient, especially humans, who face a high risk of C5a-mediated tissue destruction and death. In general, the conditions or diseases are those such as inflammatory disorders where C5a is generated in the serum proteolytically. Representative

conditions responsive to C5a analogue or polypeptide derivative of human C5a or dimer therapy include pneumonitis, adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, pulmonary inflammation or injury, chronic progressive pulmonary disease, cystic fibrosis, byssinosis, asbestos-induced inflammation, myocardial infarction, post-myocardial infarction inflammation, ischemic heart damage, hepatic cirrhosis, primary biliary cirrhosis inflammation, chronic hepatitis, pancreatitis, hemorrhagic pancreatitis, inflammatory bowel disease, colitis, ischemic brain damage, encephalitis, cranial nerve damage in memingitis, meningitis, uvetis, Purtscher's retinopathy, immune complex-mediated glomerulonephritis, renal cortical necrosis, gout, vasculitis, serum sickness, angioedema, myasthenia gravis, systemic lupus erythematosis, rheumatoid arthritis, bullous skin disease, hypersensitivity, psoriasis, endotoxin shock, sepsis, severe trauma, and burns. They can also be used therapeutically to treat patients suffering from transplant rejection, those receiving immunosuppressive therapy or massive blood transfusion, those exposed to medical devices, and those experiencing pulmonary dysfunction following hemodialysis, and leukopheresis.

The analogues, polypeptide derivatives and dimers thereof have further therapeutic utility as prophylactics, particularly in conditions caused by reperfusion, e.g., reperfusion following ischemia, and circulatory contact with medical devices, as well as to prevent transplant rejection. In this case, the C5a analogue or derivative is administered suitably prior to or substantially simultaneously with the event that is known to cause the inflammation or aggravate an existing inflammatory condition.

The C5a analogues, polypeptide derivatives and dimers thereof of the present invention can be administered by any therapeutically effective route for a proteinaceous pharmaceutical, e.g., parenterally, intranasally, rectally or buccally, in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants and vehicles as desired. The term "parenteral" embraces delivery modes such as subcutaneous, intravenous, intramuscular, instrasternal, intra-arterial injection and infusion techniques.

Dosage amounts of the C5a analogues or polypeptide derivatives (and dimers) of the present invention may be varied to achieve the desired therapeutic response for a particular patient. This will depend, for instance, on the activity of the particular antagonist, the mode of administration, the severity of the condition being treated, as well as the medical condition of the patient. The determination of a therapeutically effective dosage amount for a given condition and patient is within the level of skill in the art. In general, dosage levels

of from about 1 ug to 100 mg per kilogram of body weight per day are administered daily to the mammalian host. Preferred dosage levels range from about 0.1 mg/kg to about 20 mg/kg of body weight per day. The C5a analogue is administered to the patient as a single continuous dose over a prolonged period of time. However, the total effective dosage may be divided into multiple doses, e.g., two to four separate doses per day, if desired.

The C5a analogues or polypeptide derivatives (and dimers) of the present invention can be formulated into compositions using both known pharmaceutically acceptable ingredients and methods of preparation. See, e.g., Remington et al., Pharmaceutical Sciences, 15th Ed., Mack Pub. (Easton, PA) (1975). Suitable compositions for parenteral administration comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions immediately prior to use. Representative examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols, e.g., glycerol, propylene glycol, polyethylene glycol, and suitable mixtures thereof, vegetable oils, e.g., olive oil, and injectable organic esters such as ethyl oleate. Fluidity may be maintained by various means including the use of coating materials such as lecithin, the maintenance of required particle size (in the case of dispersions), and surfactants.

The compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, dispersing agents, antibacterial and antifungal agents such as paraben, chlorobutanol, phenol and sorbic acid, isotonic agents such as sugars, sodium chloride, or agents which delay absorption such as aluminum monostearate and gelatin. The C5a receptor antagonists may be incorporated into slow or sustained release or targeted delivery systems such as polymer matrices, liposomes, and microspheres.

Injectable formulations can be sterilized by numerous means, including filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Suspensions, in addition to the C5a analogue, polypeptide derivatives or dimers thereof and any other active ingredient, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth and mixtures thereof.

Compositions for rectal or vaginal administration are usually in the form of suppositories which can be prepared by mixing the polypeptides of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which is solid at room temperature but liquid at body temperature, and therefore melts in the rectum or vaginal cavity, and releases the receptor antagonist.

Opthalmic formulations, eye ointments, powders and solutions are also included within the scope of the disclosed invention.

A further embodiment of the present invention is directed to an antibody specific to a polypeptide derivative or dimer of the present invention, wherein said antibody exhibits substantially no cross-reactivity with human C5a. The antibody may be polyclonal or monoclonal. Preferred antibodies are those which are specific to preferred polypeptide derivatives of human C5a or dimers of the present invention as mentioned above. For example, a preferred antibody of the present invention is specific to a polypeptide derivative of human C5a, which is C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys). Another preferred antibody is specific to a dimer of the present invention, wherein each of said first and second derivatives is the human C5a derivative C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys).

Polyclonal and monoclonal antibodies specific to the C5a analogues or polypeptide derivatives of human C5a and dimers of the present invention may be prepared in accordance with standard techniques. Polyclonal antibodies, for example, are raised by injecting a C5a analogue- or polypeptide derivative- or dimer-carrier protein conjugate into an animal, e.g., rabbits, goats, sheep or horses, to raise anti-C5a analogue or anti-polypeptide derivative or anti-dimer antibodies, respectively. See, e.g., A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, Oxford (1982). Monoclonal antibodies specific to the C5a analogues, polypeptide derivatives or dimers of the present invention may be prepared according to the techniques disclosed techniques disclosed in Kohler and Milstein, Nature 256:495-97 (1975). See also Peters, J. H., (eds.) Monoclonal Antibodies, Springer Verlag Berlin, Heidelberg, Germany (1992). The polyclonal and monoclonal antibodies specific to the C5a analogues, polypeptide derivatives or dimers also exhibit substantially no cross-reactivity with human C5a. By the term "substantially no cross-reactivity," it is meant that the anti-C5a analogue, anti-polypeptide derivative or anti-dimer antibodies exhibit extremely low (negligible) cross-reactivity with human C5a such that no interference by endogenously produced C5a with the assay for the instant C5a analogues or polypeptide derivatives or dimers in biological samples can be detected.

The C5a analogue-specific, polypeptide derivative-specific or dimer-specific antibodies of the present invention are particularly useful to detect and quantify circulating C5a analogue, polypeptide derivative or dimer, respectively, in a subject previously administered with same, as well as in modulating, e.g., neutralizing, the activity of the circulating C5a analogue. Thus, another embodiment of the present invention is directed to a method of modulating the activity of a polypeptide derivative of human C5a or dimer of the present invention, in a subject in need thereof, comprising the step of: administering to the subject an antibody of the present invention, preferably in the form of a pharmaceutical composition (see below). A further embodiment of the present invention is directed to a method of neutralizing the activity of a polypeptide derivative of human C5a or dimer of the present invention in a subject in need thereof, comprising the step of: administering to the subject an antibody of the present invention, preferably in the form of a pharmaceutical composition (see blow).

Circulating C5a analogue or polypeptide derivative of human C5a or dimer of the present invention can be detected in accordance with standard immunological techniques which utilize antibodies. In general, a fluid or tissue sample is obtained from the subject and then reacted with an antibody specific to the C5a analogue which was administered to the subject, under conditions suitable to allow for the detectable formation of an immune complex between the analogue and the antibody. The formation of such an immune complex is indicative of the presence of the analogue in the sample. The use of plasma or serum samples in such assays are preferred. However, tissue such as certain blood cells, e.g., PMNL's, can also be used. The presence and/or extent of reaction can be determined in a variety of methods known in the art such as radioimmunoassay, enzyme immunoassay, fluorescent immunoassay, fluorescent microscopy, etc., and the like. Qualitative and quantitative suitable immunological assay methods are disclosed in J. Butler, Immunochemistry of Solid-Phase Immunoassay, CRC Press (1991)

Assays to detect circulating C5a analogue are typically employed to monitor levels of the analogue during treatment. Thus, a further embodiment of the present invention is directed to a qualitative or quantitative assay for the determination of a polypeptide derivative of human C5a or of a dimer of the present invention in a subject, comprising the steps of:

(1) obtaining a tissue or a fluid sample from the subject, and

(2) contacting the sample with an antibody of the present invention under conditions sufficient to allow the detectable formation of an immunocomplex between the antibody and

the derivative, wherein the formation of the immunocomplex is indicative of the presence of the derivative in the subject. Such an assay may further comprise the step of quantifying the polypeptide derivative or dimer in the subject.

In addition, the antibodies of the present invention can be advantageously used in a pharmaceutical composition to modulate or neutralize the activity of the circulating C5a analog. Hence, a further embodiment is directed to a pharmaceutical composition useful in modulating the *in vivo* activity of a polypeptide derivative of human C5a or dimer of the present invention, comprising an antibody of the present invention in an amount effective to modulate the activity of the derivative, and optionally a pharmaceutically acceptable carrier. Preferred is such a pharmaceutical composition, wherein the amount of said antibody is effective to substantially neutralize the *in vivo* activity of said derivative or dimer.

The amount of antibody used will be a molar equivalent of the amount of analogue administered. The compositions may be administered to a subject parenterally. Intravenous administration is preferred especially in an emergency situation. The antibodies will be formulated in a unit dosage injectable form in association with a pharmaceutically acceptable vehicle such as saline or Ringer's solution.

The invention will be further described by reference to the following detailed examples. As will be appreciated by those of skill, the respective methods of preparing or testing described in the examples are in principle suitable for producing or testing the polypeptide derivatives, dimers, fusion proteins, DNA molecules, plasmids, vectors, transformed hosts and antibodies, respectively, of the present invention. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Example 1

Synthesis of a Gene Encoding Human C5a

The human C5a gene is synthesized by oligonucleotide coupling. The codon usage of this synthetic gene is designed for optimal expression in *E. coli*. The synthetic strategy is illustrated in Fig. 1. It entails the condensation of five fragments with the N-terminal residue changed from Thr to Met since the AUG codon gives a much higher frequency of translation initiation than any other codon. Fragment 1 encodes a Shine-Delgarno sequence and the ATG start codon of the synthetic gene. Fragments 2-5 encode the C5a gene.

Oligonucleotide Synthesis: Oligonucleotides are synthesized on a Gene Assembler (Pharmacia) by the solid phase phosphoramidite method. The fully synthesized oligonucleotides are cleaved from the solid support and deprotected by incubation with

concentrated NH_4OH for 16 h at 55°C. The oligonucleotides are then purified by preparative gel electrophoresis. The acrylamide concentration used varied from 10% for oligonucleotides greater than 70 bases to 20% for those less than 40 bases in length. Following electrophoresis, the oligonucleotides are visualized by UV shadowing and the major high molecular weight fragment is excised from the gel. The gel slice is pulverized in a test tube with a glass rod and the DNA extracted by incubation in 3.0 ml of 0.1 M triethylammonium bicarbonate (TEAB) buffer at pH 7.5 for 16 h at 37°C.

The gel remnants are removed by centrifugation and the oligonucleotides isolated by chromatography on SepPak C-18 columns (Waters Associates). The columns are pre-equilibrated by washing sequentially with 10 ml acetonitrile, 5 ml 30% acetonitrile in 50 mM TEAB and 10 ml 25 mM TEAB. The oligonucleotides are applied, washed with 10 ml 25 mM TEAB, and eluted from the columns with 5 ml 50% acetonitrile in 35.5 mM TEAB. Fractions are collected and those containing the oligonucleotides, as determined by absorbance at 260 nm, are dried in a SpeedVac (Savant).

Oligonucleotide Annealing and Coupling: Prior to annealing, each oligonucleotide is phosphorylated at the 5' end. The kinase reaction mixture contains 1 ug of oligonucleotide in a total volume of 40 ul, 77 mM TRIS at pH 7.5, containing 12 mM $MgCl_2$, 1 mM DTT (dithiothreitol) and 2 mM ATP. The reaction is initiated by the addition of 10 units of T4 polynucleotide kinase and is allowed to proceed for 40 min at 37°C. 10 ul of sterile water are added to each kination reaction and 48 ul of complimentary oligonucleotides are added, mixed and placed in a heating block at 78°C. The heating block is turned off and the mixture is allowed to cool to 30°C. The samples then are placed in a second heating block at 68°C for 10 min, and again the block is turned off and the mixture allowed to cool to 26°C. Annealed gene fragments are used to assemble the gene in phage M13mp18 (New England Biolabs). The strategy for assembling the C5a encoding gene in M13mp18 requires three rounds of ligation reactions.

Following each ligation reaction of the appropriate gene fragments into M13mp18, *E. coli* JM101 is transformed with the ligated M13 DNA. Isolation of the M13 phage from the recombinant clones is followed by sequence analysis of the construction. The final C5a gene is cloned into M13mp18 to give M13mp18/C5a(Thr1Met, 1-74). The C5a(1-74) gene is subsequently subcloned into a pB-6 vector, derived from plasmids pTZ19R and pKK223-3 both derived from Pharmacia) to yield pB-6/C5a(Thr1Met, 1-74). See Fig. 2.

Example 2

Site-directed Mutagenesis of the C5a Gene

Using the oligonucleotide-directed in vitro Mutagenesis System Version 2 (Amersham), the single stranded DNA from the C5a containing vector M13mp18/C5a(1-74) and a mutagenic oligonucleotide, site-directed mutagenesis is performed. The mutation Cys27Ser in C5a is performed using the mutagenic oligonucleotide, ACGGTGCTTCTGTTAACA (SEQ. ID. NO. 6), following the procedure provided by the manufacturer. 4 plaques are analyzed by dideoxy DNA sequencing for the correct Cys27Ser mutation. Double stranded DNA is isolated from one of the correct mutant clones and restricted with PstI and BamHI. A 230 bp fragment containing the mutation is subcloned into the pB-6 vector. The resulting plasmid, pB-6/C5a(1-74,T1M,C27S), is sequenced again via the dideoxy method to confirm the mutation.

Example 3

Cassette Mutagenesis of the C5a Gene

The plasmid pB-6/C5a(1-74,T1M,C27S) or pB-6/C5a(1-74,T1M,) is restricted with EcoRI and HindIII, and subcloned in the vector pWCB, also restricted with the same enzymes, to yield plasmids pWCB112, containing the gene encoding for C5a(1-74,T1M,C27S), and pWCB100, containing the gene encoding for C5a(1-74,T1M), respectively. Then, the plasmids are used in cassette mutagenesis to make a series of new genes. Oligonucleotides used in cassette mutagenesis are made with an Applied Biosystems 381A DNA Synthesizer, using solid phase phosphoramidite chemistry according to the instructions of the manufacturer.

10 ul TE, containing about 60 ug of pWCB112, are mixed with 6 ul containing 60 U of PVUII and 3 ul containing 60 U of HindIII (New England Biolabs) and 10 ul of 10 x High Salt buffer (1M NaCl, 0.5 M Tris/HCl at pH 7.5, 0.1 M $MgCl_2$, 10 mM DTT) and 71 ul ddH_20 for a total volume of 100 ul. This solution is incubated at 37°C for 16 hours. The thus-linearized vector of about 4.5 Kb is purified by preparative electrophoresis using a 1% agorose gel, followed by electroelution of the DNA fragment from the excised agarose gel slice. The recovered DNA fragment is transferred to an Eppendorf tube, 1 ml of absolute ethanol is added and the tube centrifuged for 10 min at 14,000 rpm in an Eppendorf centrifuge. The DNA pellet is dried under vacuum and subsequently dissolved in 45 ul TE buffer (10 mM Tris/HCl at pH 7.4 containing 1 mM EDTA) yielding pWCB112/A.

Single stranded oligonucleotides a 35bp-sequence, 5'CTGCGTGCTAACATCTCTCACAAAGACATGTGCTA3' (SEQ. ID. NO. 7), and a 39 bp-sequence,

5'AGCTTAGCACATGTCTTTGTGAGAGATGTTAGCACGCAG3' (SEQ. ID. NO. 8), are purified by preparative electrophoresis on a 8% polyacrylamide gel. Following electrophoresis, the oligonucleotides are visualized by UV shadowing and the appropriate fragment excised from the gel. The gel slice is pulverized in a test tube with a glass rod and the DNA extracted by incubation in 3.0 ml of 0.1 M TEAB buffer at pH 7.5 for 16 h at 37°C. The gel remnants are removed by centrifugation and the oligonucleotides isolated by chromatography on SepPak C-18 columns (Waters Associates). The columns are pre-equilibrated by washing sequentially with 10 ml acetonitrile, 5 ml 30% acetonitrile in 50 mM TEAB and 10 ml 25 mM TEAB. The oligonucleotides are applied, washed with 10 ml 25 mM TEAB, and eluted from the columns with 5 ml 50% acetonitrile in 35.5 mM TEAB.

Fractions are collected and those containing the oligonucleotides, as determined by absorbance at 260 nm, are dried in a SpeedVac (Savant). Annealing of the 35 and 39 bp oligonucleotides to form double stranded DNA for ligation into the restricted vector pWCB112/A is performed by mixing equal amounts of each oligonucleotide with Klenow Buffer, which contains 0.05 M Tris/HCl at pH 7.6 containing 0.01 M $MgCl_2$, heating the sample to 95°C for 10 min and subsequent cooling to room temperature over a 2 hour period. The double- stranded DNA is ligated into the restricted vector pWCB112 using a 3-fold excess of insert over vector with 1 ul, 2 U, of T4 DNA ligase (BRL). The reaction is run for 17 hours at 4°C.

Using essentially the same technique, a number of molecules are prepared merely by using different oligonucleotides, and either pWCB100 or pWCB112. They are set forth below in Table 2.

TABLE 2

C5a analogues produced by cassette mutagenesis of the C5a(1-74) or C5a(1-74,C27S) gene

Analogue No.	oligonucleotide sequences used and encoded C5a analogue
1.	C5a(1-64,T1M,C27S,N64C)
	CTGCGTGCTTGCTA (SEQ. ID. NO. 9)
	AGCTTAGCAAGCACGCAG (SEQ. ID. NO. 10)
2.	C5a(1-65,T1M,C27S,I65C)
	CTGCGTGCTAACTGCTA (SEQ. ID. NO. 11)
	AGTTAGCAGTTAGCACGCAG (SEQ. ID. NO. 12)
3.	C5a(1-66,T1M,C27S,S66C)

CTGCGTGCTAACATCTGCTA (SEQ. ID. NO.13)
AGCTTAGCAGATGTTAGCACGCAG (SEQ. ID. NO. 14)

4. C5a(1-67,T1M,C27S,H67C)
CTGCGTGCTAACATCTCTTGCTA (SEQ. ID. NO. 15)
AGCTTAGCAAGAGATGTTAGCACGCAG (SEQ. ID. NO. 16)

5. C5a(1-68,T1M,C27S,K68C)
CTGCGTGCTAACATCTCTCACTGCTA (SEQ. ID. NO. 17)
AGCTTAGCAGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 18)

6. C5a(1-69,T1M,C27S,D69C)
CTGCGTGCTAACATCTCTCACAAATGCTA (SEQ. ID. NO. 19)
AGCTTAGCATTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 20)

7. C5a(1-70,T1M,C27S,M70C)
CTGCGTGCTAACATCTCTCACAAAGACTGCTA (SEQ. ID. NO. 21)
AGCTTAGCAGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 22)

8. C5a(1-71,T1M,C27S,Q71C)
CTGCGTGCTAACATCTCTCACAAAGACATGTGCTA (SEQ. ID. NO. 23)
AGCTTAGCACATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 24)

9. C5a(1-72,T1M,C27S,L72C)
CTGCGTGCTAACATCTCTCACAAAGACATGCAATGCTA (SEQ. ID. NO. 25)
AGCTTAGCATTGCATGTCTTTGTGAGAGATGTTAGCACGCAG(SEQ ID. NO. 26)

10. C5a(1-73,T1M,C27S,G73C)
CTGCGTGCTAACATCTCTCACAAAGACATGCAACTGTGCTAS(SEQ. ID. NO.27)
AGCTTAGCACAGTTGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 28)

11. C5a(1-74,T1M,C27S,Q71C)
CTGCGTGCTAACATCTCTCACAAAGACATGTGCCTGGGTCGTTA (SEQ. ID. NO. 29)
AGCTTAACGACCCAGGCACATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 30)

12. C5a(1-73,T1M,C27S,Q71C)
CTGCGTGCTAACATCTCTCACAAAGACATGTGCCTGGGTTA (SEQ. ID. NO. 31)
AGCTTAACCCAGGCACATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 32)

13. C5a(1-72,T1M,C27S,Q71C)
CTGCGTGCTAACATCTCTCACAAAGACATGTGCCTGTA (SEQ. ID. NO. 33)
AGCTTACAGGCACATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 34)

14. C5a(1-74,T1M,R74C)
CTGCGTGCTAACATCTCTCACAAAGACATGCAACTGGGTTGCTA (SEQ. ID. NO. 35)
AGCTTAGCAACCCAGTTGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 36)

15. C5a(1-71,T1M,C27S)
CTGCGTGCTAACATCTCTCACAAAGACATGCAATA (SEQ. ID. NO. 37)

AGCTTATTGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 38)

16. C5a(1-71,T1M,C27S,Q71D)

CTGCGTCCTAACATCTCTCACAAAGACATGGACTA (SEQ. ID. NO. 39)

AGCTTAGTCCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 40)

17. C5a(1-71,T1M,C27S,Q71S)

CTGCGTGCTAACATCTCTCACAAAGACATGTCTTA (SEQ. ID. NO. 41)

AGCTTAAGACATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 42)

18. C5a(1-71,T1M,C27S,Q71H)

CTGCGTGCTAACATCTCTCACAAAGACATGCACT A (SEQ. ID. NO. 43)

AGCTTAGTGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 44) 19.

C5a(1-71,T1M,C27S,Q71R)

CTGCGTGCTAACATCTCTCACAAAGACATGCGTTA (SEQ. ID. NO. 45)

AGCTTAACGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 46)

20. C5a(1-71,T1M,C27S,Q71L)

CTGCGTGCTAACATCTCTCACAAAGACATGCTGTA (SEQ. ID. NO. 47)

AGCTTACAGCATGTCTTTGTGAGAGATGTTAGCACGCAG (SEQ. ID. NO. 48)

21. C5a(1-71,T1M, C27S, H67F, Q71C)

CTGCGTGCTAACATCTCTTTCAAAGACATGTGCTA (SEQ. ID. NO. 49)

AGCTTAGCACATGTCTTTGAAAGAGATGTTAGCACGCAG (SEQ. ID. NO. 50)

Analogue Nos. 10-20 are agonists, and are outside the scope of the present invention. They are included for purposes of comparison. The preparation of the dimeric form of analogue No. 8 is described in Example 5c, below. It is designated Analogue No. 22.

The complete nucleotide sequence of the polynucleotide encoding C5a analogue No. 21 is set forth in Table 3 below.

TABLE 3

GAA-TTC-CCA-CTC-AAA-ATA-AGG-AGG-AAA-AAA-AA

ECOR1

ATG-CTG-CAG-AAG-AAA-ATC-GAA-GAA-ATC-GCT

START

GCT-AAG-TAC-AAA-CAC-TCT-GTT-GTT-AAA-AAA

TGC-TGC-TAC-GAC-GGT-GCT-TCT-GTT-AAC-AAC

HPA1

GAC-GAA-ACT-TGC-GAA-CAG-CGT-GCT-GCT-CGT

ATC-TCT-CTG-GGC-CCG-CGT-TGC-ATC-AAA-GCA

APA1

TTC-ACT-GAA-TGC-TGC-GTT-GTT-GCT-TCT- CAG
PVU11
CTG-CGT-GCT-AAC-ATC-TCT-TTC-AAA-GAC-ATG

TGC-TAA-GCT-T (SEQ. ID. NO. 51)
HIND111

However, those skilled in the art will appreciate that many polynucleotides can be prepared encoding the identical C5a analogue, due to the degeneracy of the genetic code. See, e.g., Watson et al., Recombinant DNA, 2nd Ed., Freeman, N.Y. (1993).

Example 4

Expression of C5a and C5a Analogues in E.coli

To achieve expression, the synthetic genes for the C5a analogues set forth in Table 1 are subcloned in the pWCB vector, the modified expression vector pKK223-3 (Pharmacia) having a BamH1 site deleted and a PvuII site changed to a PvuI site, and containing the isopropyl-thio-beta-D-galactoside (IPTG)-inducible tac-promotor, and an ampicillin resistance gene. *E. coli* strain LCIQ, is a derivative of strain LC137 (lon, htpR) disclosed in Goff et al., Proc. Natl. Acad. Sci USA *81*:6647-6651 (1984). It contains an F' factor encoding lacIQ, from strain DH5alpha F'IQ (BRL laboratories), and is the host for the expression plasmids. *E. coli* LCIQ containing the appropriate expression plasmid is grown at 30°C in LB broth until an OD_{550} of 1 is reached. The culture is induced for 3 hours with IPTG at 2.5 mM final concentration. The cells are harvested by centrifugation and stored at -80°C until use.

Example 5a

Refolding and Purification of C5a and C5a Analogues

Recombinant protein is isolated from the frozen *E. coli* cell paste aliquots from Example 4 after thawing in a buffer containing 6 M guanidinium hydrochloride (5:1 v:w, buffer:cell paste). The cells are then disrupted by sonication, and the product dialyzed overnight against a 50 mM Tris/HCl buffer at pH 8.0 containing either 1 mM cysteine and 1 mM cystine or 1 mM reduced/oxidized glutathione to promote renaturation. The dialysate is then acidified to pH 3 by the addition of 6 N HCl. The precipitate is removed by centrifugation and the supernatant is purified on a DeltaPak C18,100 Å, 15 micron, reverse phase HPLC column (Waters) using a linear gradient from 25% to 35% acetonitrile in water in the presence of 0.1% TFA over 30 min. The major peak eluting from the column at about 28% acetonitrile is collected and lyophilyzed. This fraction contained the gluthathione

adduct of recombinant C5a analogue (adducts of analogues 5, 7, 8, 9, and 21 in Table 1), or the cysteine-adduct of the C5a analogue (the adduct of analogue No. 8, Table 1).

Approximately 0.002 mmoles of the cysteine-adduct of the C5a analogue gene product are dissolved in 50 ml 0.1 M Tris buffer at pH 7.4. 0.02 mmoles of DTT are added. After 4 hours, about 80% of the C-terminal cys-cys linkages are converted to the free cysteine and the product purified on a C4, 15 micron, 300 Å, reverse phase column (Alltech) using a linear gradient from 25 to 35% acetonitrile in water in the presence of 0.1% TFA for 30 min. Fractions containing the product eluting at about 29% acetonitrile are lyophilized and then stored at 4°C, dessicated under vacuum.

Example 5b

Refolding and Purification of C5a and C5a Analogue

Recombinant protein is isolated from the frozen cell *E. coli* paste aliquots from Example 4 after thawing in a buffer containing 6 M guanidinium hydrochloride (5:1 v:w, buffer:cell paste). The cells are then disrupted by sonication and the product is diluted twenty-fold with 100 mM Tris/HCl buffer at pH 7.4 containing 1 mM reduced/0.01 mM oxidized glutathione. After 4 h, the solution is acidified to pH 3 by the addition of 6 N HCl. The resulting precipitate is removed by centrifugation and the supernatant is purified on a DeltaPak C18, 100 Å, 15 micron, reverse phase HPLC column (Waters) using a linear gradient from 25 to 35% acetonitrile in the presence of 0.1% TFA over 30 min. The major peak eluting from the column at about 30% acetonitrile is collected and lyophilized. The thus-isolated C5a analogue has a C-terminal cysteine having a reduced thiol group.

Example 5c

Formation of C5a Analogue Dimers

C5a analogues having in their C-terminal region a free thiol group are converted from the monomeric form (after refolding as described in Example 5b) to a dimeric form.

The recombinant protein is isolated from the frozen *E. coli* paste aliquots from Example 4 after refolding according to Example 5b using a 1 mM reduced/0.01 mM oxidized glutathione mixture in 100 mM Tris/HCl at pH 7.4. After 4 h, the solution is acidified to pH 3 by the addition of 6 N HCl. The resulting precipitate is removed by centrifugation and the supernatant absorbed on a SP-Spherodex\ ion exchange column, equilibrated with 25 mM buffer at pH 7.0. After washing the column with 25mM Tris at pH 7.0, the C5a analogue is eluted from the column with 25 mM Tris at pH 7.0, containing 0.75 M NaCl. The partially purified C5a analogue is brought to pH 3.0 with formic acid, and diluted with distilled water to achieve a protein solution having a conductivity of about 45 mS/cm, and absorbed to a

SP-High Performance\ ion exchange column equilibrated in 50 mM formic acid at pH 3.5, containing 0.6 M NaCl. C5a analogue is eluted from the column using a linear gradient from 0.6-1.0 M NaCl in 50 mM formic acid buffer at pH 3.5.

The major peak eluting from the column at about 0.725 M NaCl is collected. The thus-isolated C5a analogue has a C-terminal cysteine having a reduced thiol group. Adjustment of the pH to 7.0 with a 25% aqueous ammonia solution and storage of the solution resulted in a conversion of the molecule to its dimeric form. At pH 7.0 and a protein concentration of about 0.3-0.6 mg/ml and storage at 4-8°C, the conversion is at least 80% completed in 2 days. The dimeric form of the C5a analogue is finally purified on a DeltaPak C18, 100Å, 15 micron, reverse phase HPLC column (Waters) using a linear gradient from 25% to 40% acetonitrile in the presence of 0.1% TFA over 30 min. The major peak eluting from the column at about 33% acetonitrile is collected and lyophilized. The thus-isolated molecule is a dimer of the C5a analogue produced by the *E. coli* expression system.

Example 6

Receptor Binding Assay

C5a and C5a receptor antagonists are tested for their affinity for the C5a receptor. Binding of [125I] BH-labelled C5a, prepared as described in Harris et al., J. Receptor Res. *11*:115-128 (1991), to PMNL membranes is measured as described in Rollins et al., J. Biol. Chem. *263*:520-526 (1988), with modifications as described in Braunwalder et al., Mol. Immunol. *29(11)*:1319-1324 (1992). PMNLs are resuspended in Hanks balanced salt solution, without Ca^{++} and Mg^{++} and which contained 10 mM HEPES at pH 7.3, 2.5 mM $MgCl_2$, 100 units/ml DNAse I, 0.1 mM PMSF, 10 ug/ml aprotonin and 10 ug/ml leupeptin. They are then equilibrated at 400 psi for 20 min at 4°C in a nitrogen cavitation bomb. After evacuation into 3 volumes 0.5 M $KHCO_3$ containing 25 mM EDTA and the protease inhibitors listed above, the gelatinous material is removed with forceps and the mixture is centrifuged at 400 x g for 10 min at 4°C. The resulting supernatant is centrifuged at 50,000 x g for 60 min at 4°C. The pellets from the aliquots representing 200 x 10^6 cells are stored at -70°C. For binding studies, these membranes are resuspended at an equivalent of 20 x 10^6 cells/ml in 50 mM HEPES at pH 7.3, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1% bacitracin and 0.5% BSA. After further 1:75 dilution with the same buffer, 400 ul of this suspension are added to duplicate tubes containing 50 ul of [125I]BH-C5a (specific activity 2200 Ci/mmol, final concentration 4.0 pM), and 50 ul buffer or C5a analogues to be tested at various concentrations for inhibitor properties.

Nonspecific binding is determined in the presence of 10 nM unlabelled C5a. The binding reaction is initiated by the addition of the PMNL membranes and is continued for 120 min at 4°C. Bound and free radioactivity are separated by vacuum filtration through GF/C glass fiber filters (Whatman), pretreated for 90 min with 0.05% PEI (polyethyleneimine) using a Cell Harvester (Brandel, Gaithersburg, MD). Filters are washed with 3 x 5 ml of ice-cold 5 mM Tris buffer at pH 7.4 and counted in a multiwell Gamma counter (Genesys). Data are analyzed using the non-linear regression analysis program, RS/1 (Bolt, Beranek and Newman, Boston) and expressed as IC_{50} values. The results are set forth below in Table 4 as K_i values using the Cheng-Presoff equation. See Braunwalder et al., supra .

TABLE 4

Receptor binding studies of C5a analogues

C5a analogue	Receptor Binding K_i(nM)
9 Glutathione	1.85
8 Glutathione	1.7
7 Glutathione	7.2
8 Cys	2.8
9	0.9
8	0.2
7	0.4
15*	3.0
16*	8.5
17*	7.5
18*	0.15
19*	0.35
20*	0.035
21	0.1
22	0.04
C5a	0.0035

* = agonist; numbers in left column refer to Table 1.

These results demonstrate that the C5a analogues of the present invention competitively displace wild-type C5a with nanomolar K_is.

The C5a analogues of the present invention have an affinity for the C5a receptor measured as a K_i in the competitive displacement assay disclosed in Braunwalder et al., supra. (using the radioligand, [125I]Bolton-Hunter labelled C5a), of less than about 1.0×10^{-8} M, preferably less than about 2.0×10^{-9} M, and more preferably less than about 1.0×10^{-10} M.

Example 7

C5a induced Ca++ rise

Recombinant human C5a is dissolved in Hanks buffer containing 0.01% Tween-20, and all stock dilutions of C5a are made in this buffer. The acetoxymethyl ester of fura-2 (fura 2AM, Molecular Probes) is dissolved in DMSO. Neutrophils are purified from human peripheral blood by sedimentation in 6% hetastarch (HESPAN, DuPont, Waukegan, IL), followed by counter flow elutriation as described in Chapman-Kirkland et al., J. Immunol. Meth. *142*:95-104 (1991). Purified cells (2×10^6/ml) are mixed with 0.2 uM fura- 2AM and incubated for 30 min at 37°C in HEPES buffered Hanks solution without calcium or magnesium. Fifteen minutes before the assay, the cell suspension is transferred to a curvette containing a stir bar and calcium is added to 1 mM. The cell suspension is incubated with stirring at 37°C. Assays are terminated within 5 h of cell purification and a standard control response is obtained periodically to insure that the cell responses are not changing over the time of the experiment. The amount of fluorescence is determined using an SLM 8000 spectrofluorometer (SLM-Aminco Instruments, Urbana, IL). Curvettes are placed in the fluorometer and after obtaining a baseline for 10 sec, the C5a receptor antagonists to be tested for antagonistic properties are added and any change in fluoresence excitation ratio of 340 nm/380 nm (emission of 510 nm) is measured. Forty seconds after analogue addition, a challenge dose of C5a is added to a final concentration of 100 pM and the resulting change in excitation ratio is measured.

IC_{50} values are used as a measure of antagonist potency. These values are defined as the concentration of C5a analogue needed to reduce the calcium rise response of the 100 pM C5a challenge dose by 50%. EC_{50} values are used as a measure of agonist potency. EC_{50} is defined as that concentration of C5a analogue that elicited 50% of the maximum calcium rise response produced by the analogue. The results are set forth below in Table 5.

TABLE 5

C5a induced calcium rise studies on C5a analogues

analogue	Calcium Rise (nm)	
	IC_{50} (antagonist)	EC_{50} (agonist)
9 glutathione	1000	NM(not measurable)
8 glutathione	2000	NM
7 glutathione	2000	NM
8 cysteine	105	NM
9	43	NM
8	14	NM
7	54	NM
15	NM	90
16	NM	310
17	NM	120
18	NM	150
19	NM	40
20	NM	75
21	6	NM
22	10	NM
C5a	NM	0.07

Ca5 analogue No. 7 is tested up to a concentration of 1.0×10^{6} M. C5a analogue No. 8 is tested up to a concentration of 3.0×10^{-6} M, analogue No. 9 is tested up to a concentration of 1.5×10^{6} M, and analogue No. 21 is tested up to a concentration of 8.0×10^{-7} M. Agonist activity is not detected in all cases. The results, analyzed collectively with those set forth in Table 2, above, suggest that the analogues of the present invention function as competitive inhibitors of C5a. They demonstrate that the C-terminus of the analogues should be an uncomplexed cysteine or a cysteine residue which is complexed through a disulfide linkage with another C5a analogue of the present invention, to achieve the highest potency.

Example 8

Rabbit Dermal Model of Inflammation

All experiments are performed on male New Zealand White rabbits weighing 2.5 -3.0 kg. The backs of the rabbits are shaved and 40 - 50 skin sites are designated with markers of different colors. Different stimuli (i.e., C5a, C5a analogue, C5a + C5a analogue, vehicle control, etc.) are injected intradermally at 0.1 ml/site using a sterile, disposable, 26 gauge,

0.5 in. needle and 1.0 cc tuberculin syringe. I.D. injections are administered in replicates of six, roughly 45 minutes before euthanization. C5a alone is injected at a dose of 50 ng/site, and the C5a receptor antagonists are co-injected at various concentrations with the same dose of C5a. At 20 minutes prior to euthanization, 18-36 uCi of [125I]-labeled bovine serum albumin in 1.0 ml physiological saline are introduced into the systemic circulation via the marginal auricular vein. At 45 minutes, the rabbit is euthanized with an I.V. overdose of sodium pentobarbital. A 5.0 ml sample of peripheral blood is secured via cardiac puncture, centrifuged at 2000 rpm for 10 minutes, and 1.0 ml of plasma is collected and used as a reference to determine the amount of [125I] in the plasma. After death, the dorsal skin is excised and pinned to a wooden dissecting board. Blood in the major vasculature of the skin is manually expressed toward the periphery. This procedure reduces variation among skin sites and decreased background radioactivity. Inflammatory lesions are then punched out of the skin with the aid of a 15 mm cork borer and mallet and deposited in 12 x 75 mm polystyrene tubes. Injection sites are then analyzed for their radioactive content using a Gamma Counter (Genesys). The amount of [125I]-bovine serum albumin (BSA) that exuded from the blood vessels and which is localized at the inflammatory sites is found to be directly proportional to the degree of enhancement in vascular permeability. The ID_{50} value of the C5a analogue is the dose of that C5a analogue causing a 50% reduction in the radioactivity produced by 50 ng C5a co-injected at the same site.

C5a analogue No. 8 (in Table 1) is found to possess an ID_{50} of 70 ng/site, and does not cause a pro-inflammatory reaction at the dose of 175 ng/site. This result demonstrates that the analogue is an antagonist *in vivo* and does not exhibit agonist properties *in vivo*.

Example 9

C5a-Induced Neutropenia in the Rabbit

All experiments are performed on male New Zealand White rabbits weighing 2.5-3.0 kg. Rabbits are anesthetized with 10 mg/kg xylazine and 50 mg/kg ketamine administered in combination intramuscularly. A 25 gauge butterfly catheter is inserted in the lateral ear vein to use for infusions. Each blood sample (0.2 ml) is collected from the central ear artery into a plastic syringe fitted with a 25 gauge, 5/8 inch needle and charged with 7.5% EDTA as an anticoagulant. Blood is immediately expressed into a microcentrifuge tubes containing 10 microliters of 7.5% EDTA. An initial arterial blood sample (#1) is obtained and immediately thereafter vehicle or the C5a analogue of Example 8 is infused intravenously (bolus injection). Twenty seconds later, a second blood sample (#2) is obtained and twenty seconds thereafter 100 ng of C5a in 0.2 ml are infused intravenously (bolus injection).

Twenty seconds later, a third blood (#3) sample is taken. Thirty minutes later, a second round of blood sample (#4)--20 seconds--C5a infusion--20 seconds--blood sample (#5) is performed. Blood samples are evaluated by automated hematologic analysis (Technicon H*1) using software specific for rabbit blood. Reductions in neutrophil counts (number per milliliter) induced by C5a(C5a-induced neutropenia, determined by comparing blood sample #3 to #2 and #5 to #4) are compared between vehicle-treated and C5a analogue-treated animals. The C5a analogue does not alter baseline neutrophil counts from normal; i.e., the C5a analogue does not exhibit agonistic (C5a-like) properties. C5a-induced neutropenia in the C5a analogue-treated rabbits is significantly ($P>0.05$) inhibited as compared to vehicle-treated rabbits by 67% and 41% at the 40-second and 30-minute C5a challenge intervals, respectively. These results demonstrate the efficacy of administering the C5a analogues systemically.

Example 10

Comparative Receptor Binding and C5a Induced Calcium Rise of C5a Analogues with the Decapeptide

H-Ile-Ser-Phe-Lys-Asp-Met-Gln-Leu-Gly-Arg-OH (SEQ. ID. NO. 52).

Five C5a analogues (Nos. 5, 7, 8, 9 and 10 from Table 2) are prepared and run in C5a receptor binding and C5a receptor calcium rise assays and compared with the synthetic decapeptide disclosed in Table I (No. 14) in Or et al., J. Med. Chem. *35*:402-406 (1992). Results of this experiment are shown in Table 6 below.

Table 6

Compound	Receptor Binding	Ca^{++} Rise	
	K_i (nM)	IC_{50} (nM)	EC_{50}(nM)
Analogue 5	0.06	84	
" 7	0.35	859	
" 8	0.04	51	
" 9	0.15	621	
" 10	0.03		0.6
C5a(1-74)	0.0035		0.07
Decapeptide	5,000		2058

These results demonstrate that the C5a analogues tested possess a 14,000-10,000-fold greater binding affinity for the receptor than the decapeptide. The above data also show that the C5a analogues described are C5a receptor antagonists molecules that

exhibit substantially no agonist activity, while the decapeptide exhibits significant agonist activity.

Example 11

Preparation of Polyclonal Antibodies Specific to C5a(1-71,T1M,C27S,Q71C)

Antigen preparation

1 mg of C5a(1-71,T1M,C27S,Q71C) is conjugated to 2 mg Keyhole Limpet hemocyanin (KLH) using the Imject\ Immunogen EDC conjugation kit from Pierce Chemical Co. (Rockford, IL, USA), following the manufacturer's directions. Conjugation efficiency is followed by adding 3,500cpm of ^{125}I-C5a (New England Nuclear, Boston, MA). The final volume of the conjugate is 2.25 ml containing 0.34 mg C5a(1-71,T1M,C27S,Q71C) (0.15 mg/ml) and an estimated 0.9 mg/ml of KLH.

Production of anti-C5a(1-71,T1M,C27S,Q71C) antiserum

C5a(1-71,T1M,C27S,Q71C) conjugate (0.5 ml) is homogenized with 0.5 ml of Freund's Complete Adjuvant (Sigma Chemical Co., St. Louis, MO). Female New Zealand White rabbits, purchased from Millbrook Farms (Amherst, MA), are injected subcutaneously in two sites (0.2 ml homogenate per site) in the scapular areas. After 21 days the procedure is repeated. Further injections are carried out using Freund's Incomplete Adjuvant (Sigma); the third injection is given after a total of 55 days, and a fourth at 126 days. Blood (ca. 30 ml) is taken from the rabbits between 3 and 5 weeks after each injection, allowed to clot and the serum removed.

Peptide immobilization for antibody adsorption

C5a(1-71,T1M,C27S,Q71C) or C5a (1 mg) is conjugated to 2 mg Bovine serum albumin (BSA), using the Imject\ Immunogen EDC conjugation kit from Pierce Chemical Co. and ^{125}I-C5a to follow efficiency, as described above. The final volume of C5a(1-71,T1M,C27S,Q71C)/BSA is 2.25 ml at 0.25 mg/ml C5a(1-71,T1M,C27S,Q71C); for C5a/BSA the final volume is 2.25 ml at 0.32 mg/ml C5a. Both conjugates contained an estimated 0.9 mg/ml BSA.

The two peptide conjugates are dialysed against 0.2M sodium hydrogen carbonate buffered to pH 8.6 with sodium carbonate. For each conjugate, 2 ml of AH(aminohexyl)-Agarose gel (Sigma Chemical Co., St. Louis, MO) prewashed in the same buffer is activated by adding gluteraldehyde to a final concentration of 1% v/v and incubating for 15 min at 20°C. The gel is washed thoroughly in buffer to remove gluteraldehyde, then the conjugate solutions are added and incubated at 20°C for 1 hr. The uncoupled protein is rinsed away from the gel and remaining binding sites are blocked by

overnight incubation at 4°C with 20 ml 0.2M glycylglycine. The gel is packed into a 0.5 cm x 10 cm glass column and washed thoroughly with Dulbecco's phosphate-buffered saline pH 7.2 containing 0.1% sodium azide (PBS-A).

Affinity chromatography

Serum from rabbits immunized with C5a(1-71,T1M, C27S,Q71C) is passed through the C5a/BSA column at 2ml/hr. The absorbed antiserum emerging from the column is collected. The column is washed thoroughly with 0.5M NaCl buffered with 0.05M sodium phosphate to pH 7.2 and containing 0.1% sodium azide. Bound antibody is removed with 3M ammonium thiocyanate. The eluting antibody is detected using an in-line UV monitor reading at 280nm and set at 0.2 OD maximum deflection. On the first two passages of serum the eluting antibody is collected and immediately dialysed against PBS-A, then concentrated by ultrafiltration to around 1 mg/ml. This process is repeated several times for each serum batch. Sera are considered to be absorbed when no more protein is detected eluting from the C5a column.

The absorbed antiserum is then passed through the C5a(1-71,T1M,C27S,Q71C) immunoabsorbent column. Bound antibody is eluted with 3M ammonium thiocyanate and immediately dialysed against PBS-A, then concentrated to about 1 mg/ml.

Example 12a

Preparation of labelled antibody to detect bound C5a(1-71,T1M,C27S,Q71C)

The anti-C5a(1-71,T1M,C27S,Q71C) antibody eluted from the C5a column (i.e., antibody which cross-reacts with C5a) is conjugated to alkaline phosphatase: 1.4 mg of antibody in 1 ml of PBS is added to 5 mg (5,000 units) of alkaline phosphatase (Type VII-T, Sigma Chemical Co.). Gluteraldehyde is added to a final concentration of 0.2% v/v. The mix is incubated at 20°C for 90 mins, then dialysed overnight against PBS-A at 4°C. The buffer is changed to 0.05M Tris buffer, pH 8.0 containing 1mM magnesium chloride, and dialysed overnight at 4°C.

Example 12b

Detection of bound C5a(1-71,T1M,C27S,Q71C) via ELISA

Specifically purified rabbit anti-C5a(1-71,T1M,C27S,Q71C) at 0.57 mg/ml is diluted 1:500 in 0.1M sodium borate/boric acid, pH 8.6. ELISA plates (Maxisorp\, Nunc, Naperville, IL) are coated with 100 ul/well of this solution for 4hr at 20°C. The plates are washed three times to remove unbound material. Samples containing C5a(1-71,T1M,C27S,Q71C) or standard preparations of C5a(1-71,T1M,C27S,Q71C), suitably diluted PBS-A + 1% BSA (PBS/BSA), are added to the wells in 100 ul. for 4hr at 20°C.

Labelled antibody is added at 1:3000 in 100 ul PBS/BSA and incubated overnight at 4°C. The plates are washed and then enzyme substrate (for alkaline phosphatase, p-nitrophenyl phosphate (Sigma Chemical Co.) at 1 mg/ml in 10% v/v diethylamine pH 9.8) is added. Color development is allowed to proceed at 20°C in the dark for about 5hrs. The plates are read at 405nm using a Biomek 1000 (Beckman Instruments, CA, USA).

Using the same conditions described above, a standard curve of C5a(1-71,T1M,C27S,Q71C) is constructed (data not shown).

Example 12c

Specificity of affinity purified specific anti-C5a(1-71,T1M,C27S,Q71C)

C5a(1-71,T1M,C27S,Q71C) or C5a is used to coat microtiter plates at 1 ug/well in 100 ul coating buffer for 4hr at 20°C. The plates are washed and serial dilutions of the antibody eluted from C5a(1-71, T1M, C27S, Q71C) after absorption on C5a are made into the plate wells in 100 ul PBS/BSA. After 4hr incubation at 20°C, the plates are washed again. Binding of rabbit antibody is detected with goat anti-rabbit/Horseradish peroxidase (Pierce Chemical Co.) at 1:1000 in PBS/BSA, 100 ul/well. After incubating with the second antibody for 4hr at 20°C, the plates are washed and Horseradish peroxidase activity demonstrated with 2,2'-Azinobis(3-ethylbenzothiazoline)-6 sulfonic acid diammonium salt ABTS substrate (Pierce Chemical Co.). After 30min development, the color is read at 405nm.

Example 13

Measurement of C5a(1-71, T1M,C27S,Q71C) in rabbit plasma samples

Blood is sampled into heparin coated tubes from two anesthetized rabbits (#1 and #2) which are then injected intravenously with C5a(1-71,T1M,C27S,Q71C). Blood is collected after a further 30min interval. A further injection of C5a(1-71,T1M,C27S,Q71C) is then given and blood is again collected after 30min. This is repeated 4 more times. The samples are centrifuged to remove blood cells, and the plasma is removed and stored at -20°C until used in the ELISA.

The samples are diluted in PBS/BSA and quantified in the ELISA against the standard curve generated in Example 11. The increase in circulating C5a(1-71,T1M,C27S,Q71C) with time is then determined. No activity can be detected in samples taken from the two rabbits before injection of C5a(1-71,T1M,C27S,Q71C), demonstrating the specificity of the antibody. The results also demonstrate that the antibody exhibits no cross-reactivity with rabbit C5a, and that C5a(1-71,T1M,C27S,Q71C) is not a naturally occurring substance in rabbits.

Example 14

Comparison of C5a(1-71,T1M,C27S,Q71C) in rabbit circulation with standard C5a(1-71,T1M,C27S,Q71C): use of the specific anti-C5a(1-71,T1M,C27S,Q71C) antibody as an antidote for C5a(1-71,T1M,C27S,Q71C)

The plasma sample obtained from the last time point of rabbit #2 (from Example 13) is subjected to serial doubling dilutions and the slope of the curve obtained is compared with the slope of the standard curve. The two slopes are parallel, indicating that C5a(1-71,T1M,C27S,Q71C) which has been circulating in the rabbit still retained its antigenic properties and would be recognized in the ELISA in the same manner as the standard C5a(1-71,T1M,C27S,Q71C). This result also indicates that the analogue would be neutralized by this antibody if removal of the C5a(1-71,T1M,C27S,Q71C) from the circulation should become necessary.

Example 15

Preparation of monoclonal anti-C5a(1-71,T1M,C27S,Q71C)

Preparation of monoclonal antibodies is carried out in BALB/c mice using standard procedures developed by Kohler and Milstein, Nature 256:495-497 (1975). The same preparation of the C5a(1-71,T1M,C27S,Q71C) antigen (coupled to KLH) is used to immunize mice. Screening of monoclonal cell lines generated by fusing spleen cells from immunized mice with the hybridoma line P3/NSI/1-Ag4-1 (ATCC TIB 18) is carried out using C5a(1-71,T1M,C27S,Q71C)/BSA and C5a/BSA. In a direct parallel to the procedure carried out with the polyclonal rabbit antisera, those antibodies which only reacts with C5a(1-71,T1M,C27S,Q71C) and not with C5a are used as specific monoclonal anti-C5a(1-71,T1M,C27S,Q71C) antibodies. Those which recognize both are used as detection antibodies, and labelled with alkaline phosphatase.

Example 16

Construction of Gene Fusions between Human Carbonic Anhydrase II (hCAII)gene and C5a Receptor Antagonist gene

Plasmid pWCB401 contains a DNA sequence coding for the C5a receptor antagonist ((C5a(1-71, Thr1Met, Cys27Ser, Gln71Cys)). This plasmid is prepared using Seq. ID No. 23 in the cassette mutagenesis procedure described in Example 3. This plasmid then is modified by insertion of a double-stranded synthetic oligonucleotide (OL1/OL2; OL1 - 5'-AGC TGG GAT CCG ATA TCC-3' (SEQ. ID No. 53), OL2 5'-A GCT GGA TAT CGG ATC

CC-3' (SEQ. ID. No. 54)) at the unique HindIII restriction site of pWCB401 using a standard ligation protocol (Maniatis et al.) to yield plasmid pWCB401BE. This insertion adds BamHI and EcoRV restriction sites immediately downstream of the stop codon and eliminates the HindIII site. Then, two more oligonucleotides (OL3/OL4; OL3 - 5'-A GCT TTC GTT GAC GAC GAC GAT AAA AAC GGT CTG CA-3'(SEQ. ID. No. 55), OL4 - 5'G ACC GTT TTT ATC GTC GTC GTC AAC GAA-3'(SEQ. ID. No. 56)) are synthesized, phosphorylated and annealed by standard procedures (Maniatis et al., 1989). This synthesized oligonucleotide links the gene for the C5a receptor antagonist to the hCAII gene and encodes an enterokinase protease-, and hydroxylamine chemical cleavage site.

The 222 bp PstI-EcoRV fragment is recovered from pWCB401BE and is ligated in a three-way ligation experiment to the annealed double-stranded oligonucleotides OL3/OL4 and the HindIII and EcoRV digested pB0304ΔRV. pB0304ΔRV is a derivative of pB0304 (Van Heeke et al., Protein Expression and Purification 4: 265-274 (1993) and is obtained by eliminating the EcoRV restriction site in the tetracycline resistance gene. The product of the three-way ligation is designated plasmid 29A-1, and the DNA sequence surrounding the cloning junctions including the synthetic oligonucleotides is verified by standard DNA sequencing methods (Maniatis et al.). *E. coli* strains HMS174(DE3)pLysS and BL21(DE3)pLysS both from (NOVAGEN, MADISON, WI) are transformed with plasmid 29A-1 and colonies are isolated on Luria Broth agar plates supplemented with tetracycline (Maniatis et al.).

Example 17A

Construction of C5a Fusion Proteins; Determination of Expression TITERS in *E. coli*

In the course of experiments trying to exchange a fragment of the human interleukin-1β gene (modified with *E. coli*-preferred codons, obtained from British Biotechnology Limited, UK) with a fragment coding for a peptide fragment of human interleukin-1 receptor antagonist (hIL1-RA) by overlap extension PCR, an artifact translational stop codon following the codon for amino acid at position 72 is obtained. A BamHI cleavage site (GGATCC) is later introduced at the stop codon site to facilitate the construction of fusion proteins. The thus-obtained synethic gene is as follows:

DNA sequence of IL72 flanked by NcoI and GamHI restriction sites

5'CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA CTCCCAACAG
AAGAGCTTAG TAATGTCTGG TCCGTACGAG CTCAAAGCTC TGCATCTGCA
AGGCCAGGAC ATGGAACAAC AGGTTGTATT CAGCATGAGC TTCATTGAGC
CTCATGCTCT TGCATTAGGC CTGAAAGAGA AGAATCTGTA CCTCAGCTGC
GTACTGAAAG CTGCGTCTCA TATGTTGGAT CC-3' (Seq. ID No. 57)

This synthetic gene is flanked by NcoI and BamHI sites and codes for a hybrid protein composed of sequences of human interleukin-1β (hIL-1β) and human interleukin-1 receptor antagonist (hIL1-RA).

This synthetic gene is then cloned as a NcoI-BamHI fragment into plasmid pPLMu resulting in plasmid pPLMuIL72. Plasmid pPLMu is plasmid pPLmuSMCori (Buell, G. et al., Nucleic Acids Res. (1985) 13:1023-1038) with the NcoI - HindIII fragment replaced by a multiple cloning site set forth as below:

Sequence of EcoRI-Hind III fragment in pPLmuSMCori containing the ribosome binding site and multiple cloning site

5'-GAATTCTTAC ACTTAGTTAA ATTGCTAACT TTATAGATTA CAAAACTTAG
GAGGGTTTTT ACCATGGTTA CGAATTCCCG GGGATCCGTC GACCTGCAGC
CAAGCTT-3' (Seq. ID No. 58)

The encoded hybrid protein is composed - counting from its N-terminal methionine - of amino acids 1-47 of hIL-1β, followed by aminio acids 52-57 of hIL1-RA, followed by amino acids 60-71 of hIL-1β, followed by the amino acid sequence Cys Val Leu Lys Ala Ala Ser (SEQ. ID. No. 59), and a translational stop codon.

E. coli strain LC 137 (Goff, S.A. et al., Nat'l Proc. Acad. Sci., USA 81:6647-6651 (1984)) is then transformed with plasmid pPLmuIL72 carrying the compatible plasmid pcI_{857} encoding the thermolabile phage λCI_{857} repressor. Heat induction resulted in high expression of the hybrid protein as analyzed by SDS-PAGE of heat induced E. coli cells (Buell, et al., Supra). The hybrid protein encoded by plasmid pPLMuIL72 is further shortened by PCR using primers C, D and E as 3' primers;

PCR primer

Primer C

5'CTTATAGGATCCAGATTCTTCTCTTTCAGGCCTAATGCAAG (Seq. ID No. 60)

Primer D

5'CTTATAGGATCCAGAGCATGAGGCTCAATGAAG (Seq. ID No. 61)

Primer E

5'CTTATAGGATCCAGATGCAGAGCTTTGAGCTC (Seq. ID No. 62)

and Primer F

5'TATAAGTCCATGGCACCGGTTAG (Seq. ID No. 63),

as 5' primer resulting in plasmids pPLMulL33, pPLMulL53 and pPLMulL63, respectively. The sequence GAT (Asp) in all these constructions of the BamHI site is in proper reading frame with the DNA sequence coding for the hybrid protein. Any coding region cloned into this site via BamHI cleavage will be preceeded by the acid labile amino acid sequence Asp-Pro encoded by the sequence GATCCX which is partly contained in the BamHI cleavage sequence. Acid cleavage of the these fusion proteins will liberate the fusion partner through cleavage at the acid labile Asp-Pro site. The constructs in pPLMulL63 and pPLMulL53 showed the same amount of hybrid protein expression as the original pPLMulL72. No expression is observed with pPLMulL33. Expression levels are determined afters SDS-PAGE and Coomassie® (ICI, Ltd.) Brilliant Blue staining.

pPLMulL33 and PPLMulL53 are then used to construct fusion proteins with human C5a. A BamHI site coding for an asparagine (GAT) which is in proper reading frame with the following hC5a gene is introduced by primer directed PCR mutagenesis. The BamHI - HindIII-cut C5a fragment is ligated into BamHI-HindIII cut plasmids pPLMulL33 and pPLMulL53 resulting in the plasmids pPLMulL33-C5a and pPLMulL53-C5a, respectively.

Sequence of IL33-C5a flanked by NcoI and HindIII sites

```
5'-CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA  CTCCCAACAG
   AAGAGCTTAG TAATGTCTGG TCCGTACGAG CTCAAAGCTC  TGCATCTGGA
   TCCCTGCAGA AGAAAATCGA AGAAATCGCT GCTAAGTACA  AACACTCTGT
   TGTTAAAAAA TGCTGCTACG ACGGTGCTTC TGTTAACAAC  GACGAAACTT
   GCGAACAGCG TGTCGCTCGT ATCTCTCTGG GCCCGCGTTG  CATCAAAGCA
```

TTCACTGAAT GCTGCGTTGT TGCTTCTCAG CTGCGTGCTA ACATCTCTCA
CAAAGACATG TGCTAA-3' (Seq. ID No. 64)

IL53-C5a sequence flanked by NcoI and HindIII sites

5'-CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA CTCCCAACAG
AAGAGCTTAG TAATGTCTGG TCCGTACGAG CTCAAAGCTC TGCATCTGCA
AGGCCAGGAC ATGGAACAAC AGGTTGTATT CAGCATGAGC TTCATTGAGC
CTCATGCTCT GGATCCCTGC AGAAGAAAAT CGAAGAAATC GCTGCTAAGT
ACAAACACTC TGTTGTTAAA AAATGCTGCT ACGACGGTGC TTCTGTTAAC
AACGACGAAA CTTGCGAACA GCGTGTCGCT CGTATCTCTC TGGGCCCGCG
TTGCATCAAA GCATTCACTG AATGCTGCGT TGTTGCTTCT CAGCTGCGTG
CTAACATCTC TCACAAAGAC ATGTGCTAA-3' (Seq. ID No. 65)

Heat induction of *E. coli* K12 LC 137 carrying pPLMulL33-C5a and pPLMulL53-C5a reslts in high expression of the fusion protein as judged by SDS-PAGE and WESTERN analysis with anti-hC5a polyclonal antibodies.

Example 17b

Construction of a gene fusion between a fragment of human Interleukin 1β / human Interleukin 1 Receptor Antagonist and C5a Analogue or Polypeptide Derivative of human C5a

The 5' region of plasmid pPLMulL53-C5a encompassing codons 1-50 is recovered and optimized using PCR for the purpose of expressing a fusion protein with C5aRA or polypeptide derivative of human C5a. Briefly, the 5' end is adapted to facilitate cloning into the pET-series bacterial expression vectors. The cysteine codon at position 9 is replaced by a serine codon, and the 3' end of the fragment is modified to allow for the fusion with C5aRA or polypeptide derivative of human C5a and cleavage linker. The following experiments are performed:

The gene encoding the IL-1β/IL1RA fragment is recovered and adapted from plasmid pPLMulL53-C5a by PCR using standard DNA amplification conditions (Maniatis et al., 1989) and oligonucleotides designated G83 and G84 as primers (G83: 5'-C GCA AGC TTG AGG CTC AAT GAA GCT CAT-3' (Seq. ID No. 66)) (G84: 5'-GGA GAT ATA CAT ATG GCA CCG GTT AGA TCT CTG AAC AGC ACC CTT CGC-3'(SEQ ID No. 67)). The amplified fragment is digested with restriction endonucleases NdeI and HindIII and recovered from an agarose gel (fragment 1). Plasmid 29A-1 is digested with HindIII and BamHI and the fragment

containing the C5aRA or polypeptide derivative gene is recovered (fragment 2). pET9c (pET System Manual, 3rd edition, 1993, Novagen) is digested with NdeI and BamHI and used in a three-way ligation experiment with fragments 1 and 2 to construct plasmid 58A-1. In this construct, the gene coding for C5aRa or polypeptide derivative is linked to a modified fragment of the hybrid IL1 gene fragment via a linker sequence that codes for an enterokinase and hydroxylamine cleavage site. *E. coli* strains BL21(DE3)pLysS and HMS174(DE3)pLysS are transformed with p58A-1 and colonies are isolated on Luria Broth agar plates supplemented with kanamycin and chloramphenicol (Maniatis et al., 1989).

Example 18

Expression of C5a Analogue or Polypeptide Derivative of human C5a fusion proteins in E. coli

Expression experiments are performed using standard procedures for the T7/pET expression system described in Studier et al., Methods Enzymol. 185:60-89 (1990), and Van Heeke et al., Protein Expression and Purification 4:265-274 (1993). Briefly, single E. coli colonies harboring expression plasmid are grown in Luria Broth supplemented with the appropriate antibiotic for selection until the optical density (measured at 550 nm) reached about 0.6. At this point, IPTG and Zn++ are added to a final concentration of 0.4 mM and 12.5 μM, respectively. Incubation of cells is continued at either 21, 30 or 37°C for an additional 2-4 hrs. Cells are harvested by centrifugation and stored at -80° C until use.

Example 19

Hydroxylamine cleavage of C5a Analogue or Polypeptide Derivative fusion protein

Recombinant fusion protein is isolated from the frozen E. coli paste aliquots from Example 18 after thawing in a 50 mM Tris/HCl buffer, containing 1 mM EDTA at pH 8.0 (5:1 v:w, buffer:cell paste). The cells are then disrupted by sonication, and the crude extract centrifuged for 20 min at 3,000 x g. The fusion protein-containing pellet is solubilized in a solution containing 0.2 M CAPSO, 2 M NH_2OH.HCL, 6 M guanidinium hydrochloride, adjusted to pH 9.3 with LiOH (7:1 v:w, buffer:cell pellet).

The solution is incubated at 37° C for 7 hours to promote fusion protein cleavage by hydroxylamine to occur in excess of 80%, after which the pH is adjusted to 8.0 with 4 M

HCl. The solution, containing in excess of 80% hydroxylamine cleaved fusion protein, is dialyzed against 25 mM Tris.HCL at pH 7.3 overnight. The dialysate is then centrifuged at 30,000 x g for 20 min and the C5aRA- (or polypeptide derivative)-containing precipitate collected. The precipitate is solubilized in 6 M guanidinium hydrochloride (5:1 v:w, buffer:precipitate), and diluted twenty-fold with 100mM Tris/HC1 buffer at pH 7.4 containing 1mM reduced/0.01 mM oxidized glutathione as described in Example 5b.

Purification and dimerization are then performed according to the methods as described above.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application is specifically and individually indicated to be incorporated by reference.

Various modifications of the invention described herein will become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: CIBA-GEIGY AG

(B) STREET: Klybeckstr. 141

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(I) TELEX: 962 991

(ii) TITLE OF INVENTION: C5a receptor antagonists having substantially no agonist activity and methods for preparation

(iii) NUMBER OF SEQUENCES: 69

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Asp Gly Ala

1

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Gly Ala Tyr
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Leu Gln Lys Lys Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser
1 5 10 15

Val Val Lys Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu
20 25 30

Thr Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys Ile
35 40 45

Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu Arg Ala Asn

50 55 60

Ile Ser His Lys Asp Met Gln Leu Gly Arg
65 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 239 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTCTATGA CTCTGCAAAA GAAGATCGAA GAAATCGCTG CTAAGTACAA GCACTCCGTC 60

GTTAAGAAGT GTTGTTACGA TGGTGCATGC GTCAACAACG ACGAAACCTG TGAACAACGA 120

GCTGCTCGTA TTTCTCTGGG CCCTCGCTGT ATCAAGGCTT TCACTGAATG TTGTGTTGTC 180

GCTTCCCAAC TGCGCGCTAA CATTTCTCAC AAGGACATGC AACTCGGCCG CTAAAAGCT 239

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACGGTGCTTC TGTTAACA 18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TGCTA 35

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCTTAGCAC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCGTGCTT GCTA 14

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCTTAGCAA GCACGCAG 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTGCGTGCTA ACTGCTA 17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCTTAGCAG TTAGCACGCA G 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTGCGTGCTA ACATCTGCTA 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTTAGCAG ATGTTAGCAC GCAG 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTGCGTGCTA ACATCTCTTG CTA 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCTTAGCAA GAGATGTTAG CACGCAG 27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTGCGTGCTA ACATCTCTCA CTGCTA 26

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTTAGCAG TGAGAGATGT TAGCACGCAG 30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGCGTGCTA ACATCTCTCA CAAATGCTA 29

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTTACGAT TTGTGAGAGA TGTTAGCACG CAG 33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTGCGTGCTA ACATCTCTCA CAAAGACTGC TA 32

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCTTAGCAG TCTTTGTGAG AGATGTTAGC ACGCAG 36

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TGCTA 35

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCTTAGCAC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CAATGCTA 38

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTTAGCAT TGCATGTCTT TGTGAGAGAT GTTAGCACGC AG 42

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CAACTGTGCT A 41

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGCTTAGCAC AGTTGCATGT CTTTGTGAGA GATGTTAGCA CGCAG 45

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TGCCTGGGTC GTTA 44

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGCTTAACGA CCCAGGCACA TGTCTTTGTG AGAGATGTTA GCACGCAG 48

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TGCCTGGGTT A 41

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AGCTTAACCC AGGCACATGT CTTTGTGAGA GATGTTAGCA CGCAG 45

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TGCCTGTA 38

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AGCTTACAGG CACATGTCTT TGTGAGAGAT GTTAGCACGC AG 42

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CAACTGGGTT GCTA 44

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGCTTAGCAA CCCAGTTGCA TGTCTTTGTG AGAGATGTTA GCACGCAG 48

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CAATA 35

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

AGCTTATTGC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CTGCGTCCTA ACATCTCTCA CAAAGACATG GACTA 35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AGCTTAGTCC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CTGCGTGCTA ACATCTCTCA CAAAGACATG TCTTA 35

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AGCTTAAGAC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CACTA 35

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AGCTTAGTGC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CGTTA 35

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

AGCTTAACGC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CTGCGTGCTA ACATCTCTCA CAAAGACATG CTGTA 35

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

AGCTTACAGC ATGTCTTTGT GAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CTGCGTGCTA ACATCTCTTT CAAAGACATG TGCTA 35

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AGCTTAGCAC ATGTCTTTGA AAGAGATGTT AGCACGCAG 39

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 252 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GAATTCCCAC TCAAAATAAG GAGGAAAAAA AAATGCTGCA GAAGAAAATC GAAGAAATCG 60

CTGCTAAGTA CAAACACTCT GTTGTTAAAA AATGCTGCTA CGACGGTGCT TCTGTTAACA 120

ACGACGAAAC TTGCGAACAG CGTGCTGCTC GTATCTCTCT GGGCCCGCGT TGCATCAAAG 180

CATTCACTGA ATGCTGCGTT GTTGCTTCTC AGCTGCGTGC TAACATCTCT TTCAAAGACA 240

TGTGCTAAGC TT 252

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Ile Ser Phe Lys Asp Met Gln Leu Gly Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGCTGGGATC CGATATCC 18

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGCTGGATAT CGGATCCC 18

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

AGCTTTCGTT GACGACGACG ATAAAAACGG TCTGCA 36

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

GACCGTTTTT ATCGTCGTCG TCAACGAA 28

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA CTCCCAACAG AAGAGCTTAG 60

TAATGTCTGG TCCGTACGAG CTCAAAGCTC TGCATCTGCA AGGCCAGGAC ATGGAACAAC 120

AGGTTGTATT CAGCATGAGC TTCATTGAGC CTCATGCTCT TGCATTAGGC CTGAAAGAGA 180

AGAATCTGTA CCTCAGCTGC GTACTGAAAG CTGCGTCTCA TATGTTGGAT CC 232

(2) INFORMATION FOR SEQ ID NO:58

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GAATTCTTAC ACTTAGTTAA ATTGCTAACT TTATAGATTA CAAAACTTAG GAGGGTTTTT 60

ACCATGGTTA CGAATTCCCG GGGATCCGTC GACCTGCAGC CAAGCTT 107

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Cys Val Leu Lys Ala Ala Ser
1 5

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CTTATAGGAT CCAGATTCTT CTCTTTCAGG CCTAATGCAA G 41

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CTTATAGGAT CCAGAGCATG AGGCTCAATG AAG 33

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CTTATAGGAT CCAGATGCAG AGCTTTGAGC TC 32

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATAAGTCCA TGGCACCGGT TAG 23

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 316 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA CTCCCAACAG AAGAGCTTAG 60

TAATGTCTGG TCCGTACGAG CTCAAAGCTC TGCATCTGGA TCCCTGCAGA AGAAAATCGA 120

AGAAATCGCT GCTAAGTACA AACACTCTGT TGTTAAAAAA TGCTGCTACG ACGGTGCTTC 180

TGTTAACAAC GACGAAACTT GCGAACAGCG TGTCGCTCGT ATCTCTCTGG GCCCGCGTTG 240

CATCAAAGCA TTCACTGAAT GCTGCGTTGT TGCTTCTCAG CTGCGTGCTA ACATCTCTCA 300

CAAAGACATG TGCTAA 316

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 379 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CCATGGCACC GGTTAGATCT CTGAACTGCA CCCTTCGCGA CTCCCAACAG AAGAGCTTAG 60

TAATGTCTGG TCCGTACGAG CTCAAAGCTC TGCATCTGCA AGGCCAGGAC ATGGAACAAC 120

AGGTTGTATT CAGCATGAGC TTCATTGAGC CTCATGCTCT GGATCCCTGC AGAAGAAAAT 180

CGAAGAAATC GCTGCTAAGT ACAAACACTC TGTTGTTAAA AAATGCTGCT ACGACGGTGC 240

TTCTGTTAAC AACGACGAAA CTTGCGAACA GCGTGTCGCT CGTATCTCTC TGGGCCCGCG 300

TTGCATCAAA GCATTCACTG AATGCTGCGT TGTTGCTTCT CAGCTGCGTG CTAACATCTC 360

TCACAAAGAC ATGTGCTAA 379

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CGCAAGCTTG AGGCTCAATG AAGCTCAT 28

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GGAGATATAC ATATGGCACC GGTTAGATCT CTGAACAGCA CCCTTCGC 48

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

Val Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

Val Asp Asp Asp Asp Lys Asn Gly
1 5

Claims:

1. A polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, said derivative having a glycine residue as the N-terminus.

2. The derivative according to claim 1, wherein said glycine residue is in the form of an adduct.

3. The derivative according to claim 1, wherein said glycine residue is a substituent for the N-terminal amino acid residue Thr of human C5a.

4. The derivative according to claim 1, wherein said derivative comprises a C-terminal region which differs from the corresponding C-terminal region of human C5a, in that it has a cysteine residue, and is truncated at its C-terminus by at least two amino acid residues.

5. The derivative according to claim 4, having a cysteine residue as the C-terminus.

6. The derivative according to claim 4, wherein said cysteine residue is in the form of an adduct.

7. The derivative according to claim 4, which is from 64 to 72 amino acids in length.

8. The derivative according to claim 7, which is from 68 to 72 amino acids in length.

9. The dervative according to claim 8, which is from 70 to 72 amino acids in length.

10. The derivative according to claim 9, which is 71 amino acids in length.

11. The derivative of claim 10, which is C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys).

12. The derivative of claim 10, which is C5a (1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys).

13. A dimer, comprising first and second polypeptide derivative of human C5a, wherein each of said derivatives is a C5a receptor antagonist that exhibits substantially no agonist activity, and has a C-terminal cysteine residue, and wherein the cysteine residues of said first and second derivatives are linked together via a disulfide linkage, and wherein said first and second polypeptide derivative of human C5a may be the same or different, and further wherein at least one of said first and second polypeptide derivatives of human C5a is a polypeptide derivative of human C5a as defined in claim 1.

14. The dimer according to claim 13, wherein the C-terminal region of each of said first and said second derivatives differs from the corresponding C-terminal region of human C5a in that it is truncated by at least two amino acid residues.

15. The dimer of claim 14, wherein each of said first and second derivatives is the human C5a derivative C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys).

16. A fusion protein, consisting of, in the order of N-terminus to C-terminus, a fusion partner, a cleavable linker and, fused thereto, a polypeptide derivative of human C5a, wherein said polypeptide derivative is a C5a receptor antagonist that exhibits substantially no agonist activity.

17. The fusion protein according to claim 16, wherein said derivative comprises a C-terminal region which differs from the corresponding C-terminal region of human C5a, in that it has a cysteine residue, and is truncated at its C-terminus by at least two amino acid residues.

18. The fusion protein according to claim 17, wherein said derivative has a cysteine residue as the C-terminus.

19. The fusion protein according to claim 17, wherein said derivative has a glycine residue as the N-terminus.

20. The fusion protein according to claim 16, wherein said fusion partner is a polypeptide capable of directing the formation of inclusion bodies in a cell.

21. The fusion protein according to claim 20, wherein said cell is an E. coli cell.

22. The fusion protein according to claim 20, wherein said fusion partner together with said cleavable linker is a polypeptide about the amino acid length of said polypeptide derivative of human C5a.

23. The fusion protein according to claim 22, wherein said fusion partner comprises an N-terminal fragment of human IL-1β or a mutant thereof.

24. The fusion protein according to claim 23, wherein said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof and amino acid residues 52 to 57 of human IL-1RA.

25. The fusion protein according to claim 23, wherein said fusion partner consists of amino acid residues 1 to 47 of human IL-1β or a mutant thereof and amino acid residues 52 to 54 of human IL-1RA.

26. The fusion protein acccording to claim 16, wherein said linker comprises a hydroxylamine cleavage site.

27. The fusion protein according to claim 26, wherein said linker comprises, in the order of N-terminus to C-terminus, an enterokinase protease cleavage site and a hydroxylamine cleavage site.

28. The fusion protein according to claim 16, consisting of, in the order of N-terminus to C-terminus, amino acid residues 1 to 47 of human IL-1β or a mutant thereof, amino acid residues 52 to 54 of human IL-1RA, a cleavable linker comprising the amino acid sequence -Val-Asp-Asp-Asp-Asp-Lys-Asn-Gly-, and a polypeptide derivative as defined in claim 1, wherein the C-terminal residue Gly of said linker is a substituent for the N-terminal amino acid residue Thr of human C5a in said polypeptide derivative.

29. The fusion protein according to claim 28, wherein said polypeptide derivative is as defined in claim 7.

30. The fusion protein according to claim 29, wherein said polypeptide derivative is as defined in claim 11.

31. The fusion protein according to claim 29, wherein said polypeptide derivative is as defined in claim 12.

32. A DNA molecule encoding a polypetide derivative of human C5a as defined in claim 1.

33. The DNA molecule according to claim 32, wherein said derivative is as defined in claim 7.

34. The DNA molecule according to claim 33, wherein said derivative is as defined in claim 11.

35. The DNA molecule according to claim 33, wherein said derivative is as defined in claim 12.

36. A DNA molecule encoding a fusion protein as defined in claim 16.

37. The DNA molecule according to claim 36, wherein said fusion protein is as defined in claim 19.

38. The DNA molecule according to claim 37, wherein said fusion protein is as defined in claim 24.

39. The DNA molecule according to claim 37, wherein said fusion protein is as defined in claim 25.

40. The DNA molecule according to claim 37, wherein said fusion protein is as defined in claim 28.

41. A recombinant DNA molecule, comprising a promoter capable of functioning in a given host operably linked to a DNA molecule as defined in claim 32.

42. A recombinant DNA molecule, comprising a promoter capable of functioning in a given host operably linked to a DNA molecule as defined in claim 36.

43. A recombinant plasmid compatible with a given host, comprising a recombinant DNA molecule according to claim 32.

44. A recombinant plasmid compatible with a given host, comprising a recombinant DNA molecule according to claim 36.

45. A recombinant vector compatible with a given host, comprising a recombinant DNA molecule according to claim 32.

46. A recombinant vector compatible with a given host, comprising a recombinant DNA molecule according to claim 36.

47. A recombinant host, stably transformed with a recombinant DNA molecule according to claim 32.

48. The recombinant host according to claim 47, selected from the group consisting of bacterial, yeast, fungal, insect, mammalian and plant cells.

49. The recombinant host according to claim 48, which is *E. coli*.

50. A recombinant host, stably transformed with a recombinant DNA molecule according to claim 36.

51. The recombinant host according to claim 50, selected from the group consisting of bacterial, yeast, fungal, insect, mammalian and plant cells.

52. The recombinant host according to claim 51, which is *E. coli.*

53. A method of preparing a biologically active polypeptide derivative of human C5a as defined in claim 1, comprising the steps of:
(1) culturing *E. coli* cells stably transformed with a DNA molecule encoding said polypeptide derivative under conditions suitable to cause expression of the DNA molecule;
(2) contacting the thus-cultured cells with a denaturing and solubilizing agent to produce said polypeptide derivative in denatured form; and
(3) mixing the thus-denatured polypeptide derivative with a solution containing a reducing agent and an oxidizing agent in a molar ratio of the reducing agent to the oxidizing agent by weight of at least about 100:1 under suitable conditions to produce the polypeptide derivative in biologically active form.

54. The method according to claim 53, wherein said DNA molecule encodes the polypetide derivative in the form of a fusion protein.

55. The method according to claim 54, further comprising the step of cleaving the thus-expressed fusion protein prior to said step of mixing.

56. The method according to claim 54, wherein said DNA molecule encodes a polypeptide derivative of human C5a, which is from 64 to 72 amino acid residues in length.

57. The method according to claim 56, wherein the polypeptide derivative is C5a (1-71, Thr1Gly, Cys27Ser, Gln71Cys).

58. A method according to claim 58, wherein the polypeptide derivative is C5a (1-71, Thr1Gly, Cys27Ser, His67Phe, Gln71Cys).

59. A method of preparing a biologically active polypeptide derivative of human C5a, wherein said derivative is a C5a receptor antagonist that exhibits substantially no agonist activity, comprising the steps of:
(1) culturing host cells stably transformed with a recombinant DNA molecule encoding said polypeptide derivative in the form of a fusion protein wherein said culturing is conducted

under conditions suitable to cause expression of said fusion protein in the form of inclusion bodies;
(2) isolating the inclusion bodies containing the fusion protein from the thus-cultured host cells;
(3) contacting the thus-isolated inclusion bodies with a denaturing and solubilizing agent to produce the fusion protein in denatured form;
(4) cleaving the thus-isolated fusion protein so that the polypeptide derivative of human C5a can be obtained; and
(5) mixing the thus-cleaved fusion protein with a solution containing a reducing agent and an oxidizing agent in a molar ratio of the reducing agent to the oxidizing agent by weight of at least about 100:1 under suitable conditions to produce the polypeptide derivative in biologically active form;
wherein steps 3 and 4 are carried out simultaneously or consecutively.

60. The method according to claim 59, wherein said mixing is conducted at a pH of from about 6.5 to about 7.5.

61. The method according to claim 59, wherein said mixing is conducted for a period of time from about 1/2 hour to about 4 hours.

62. The method according to claim 59, wherein the redox couple is reduced glutathione/oxidized glutathione.

63. The method according to claim 59, wherein said host cells are *E. coli* cells.

64. The method according to claim 59, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 16.

65. The method according to claim 64, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 19.

66. The method according to claim 65, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 22.

67. The method according to claim 66, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 23.

68. The method according to claim 67, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 24.

69. The method according to claim 67, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 25.

70. The method according to claim 59, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 28.

71. The method according to claim 70, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 29.

72. The method according to claim 71, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 30.

73. The method according to claim 71, wherein said recombinant DNA molecule encodes a fusion protein as defined in claim 31.

74. An antibody specific to the polypeptide derivative as defined in claim 1 or to a domer as defined in claim 13, wherein said antibody exhibits substantially no cross-reactivity with human C5a.

75. An antibody according claim 74, wherein said antibody is polyclonal.

76. An antibody according to claim 74, wherein said antibody is monoclonal.

77. An antibody according to claim 74, specific to the derivative as defined in claim 11.

78. An antibody according to claim 74, specific to a dimer as defined in claim 14.

79. An antibody according to claim 78, specific to the dimer as defined in claim 15.

80. A pharmaceutical composition useful in the treatment of a C5a-mediated disease or inflammatory condition in a mammal inclusive man, comprising a therapeutically effective amount of a polypeptide derivative of human C5a as defined in claim 1 or of a dimer as defined in claim 13, and optionally a pharmaceutically acceptable carrier.

81. A pharmaceutical composition according to claim 80, wherein said derivative is as defined in claim 11, or said dimer is as defined in claim 15.

82. A pharmaceutical composition useful in modulating the *in vivo* activity of a polypeptide derivative of human C5a as defined in claim 1, comprising
an antibody as defined in claim 74 in an amount effective to modulate the activity of the derivative, and optionally a pharmaceutically acceptable carrier.

83. The pharmaceutical composition according to claim 82, wherein the amount of said antibody is effective to substantially neutralize the *in vivo* activity of said derivative.

84. A method of treating a C5a-mediated disease or inflammatory condition in a mammal, comprising the step of administering a pharmaceutical composition according to claim 80 to a mammal inclusive man in need thereof.

85. A method of reducing C5a-mediated inflammation in a mammal inclusive man, comprising the step of administering a pharmaceutical composition according to claim 80 to said mammal at a time relative to a complement activation-causing or aggravating event sufficient to reduce the inflammation.

86. A method of modulating the activity of a polypeptide derivative as defined in claim 1 or of a dimer as defined in claim 13, in a subject in need thereof, comprising the step of: administering to the subject a pharmaceutical composition according to claim 82.

87. A method of neutralizing the activity of a polypeptide derivative as defined in claim 1 or of a dimer as defined in claim 13 in a subject in need thereof, comprising the step of:

administering to the subject a pharmaceutical composition according to claim 83.

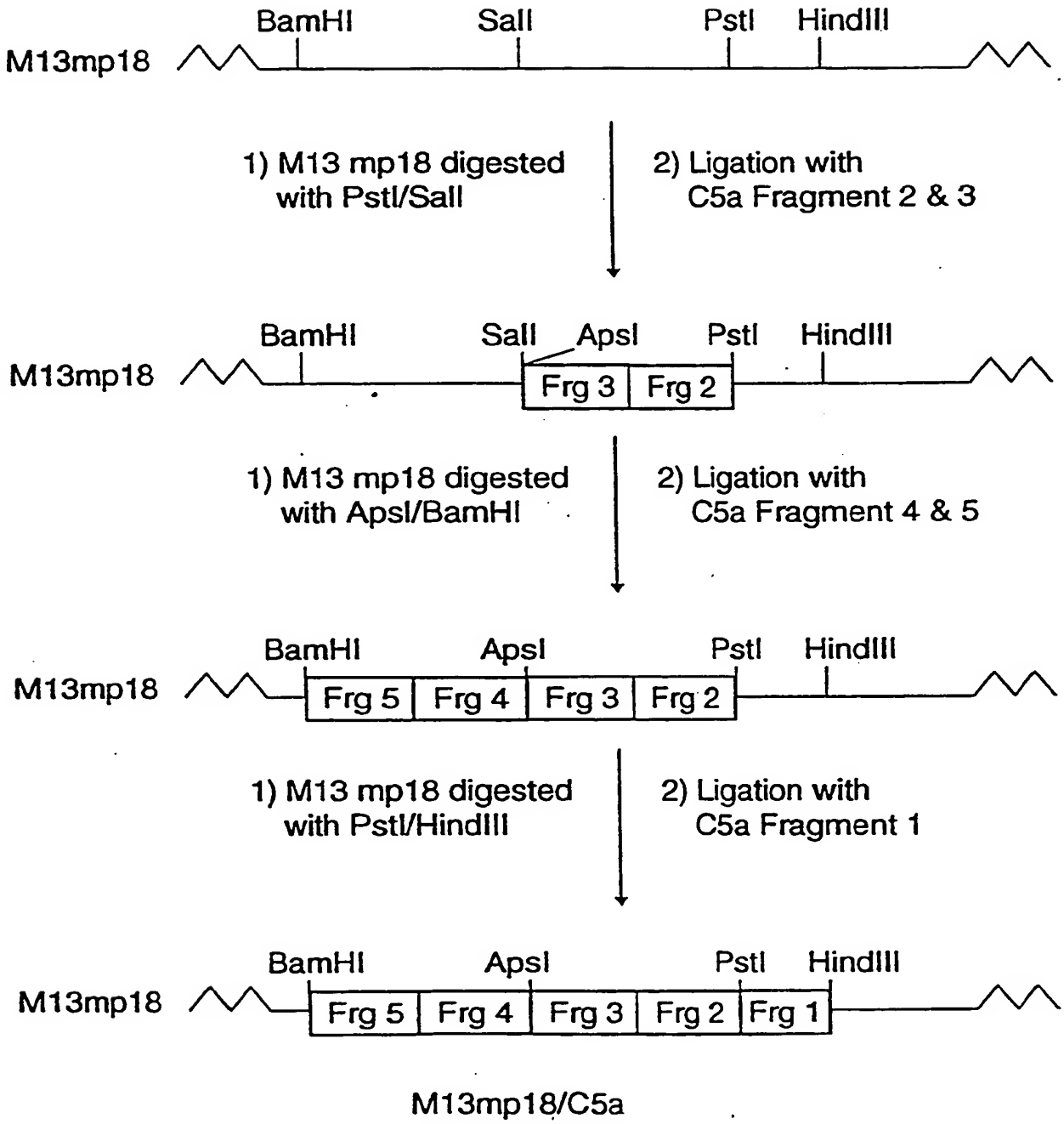
88. A qualitative or quantitative assay for the determination of a polypeptide derivative as defined in claim 1 in a subject, comprising the steps of:
(1) obtaining a tissue or a fluid sample from the subject, and
(2) contacting the sample with an antibody according to claim 74 under conditions sufficient to allow the detectable formation of an immunocomplex between the antibody and the derivative, wherein the formation of the immunocomplex is indicative of the presence of the derivative in the subject.

89. An assay according to claim 88, further comprising the step of quantifying the derivative in the subject.

90. A polypeptide derivative according to claim 1 or a dimer according to claim 13 for use in the therapeutic treatment of a mammal inclusive man.

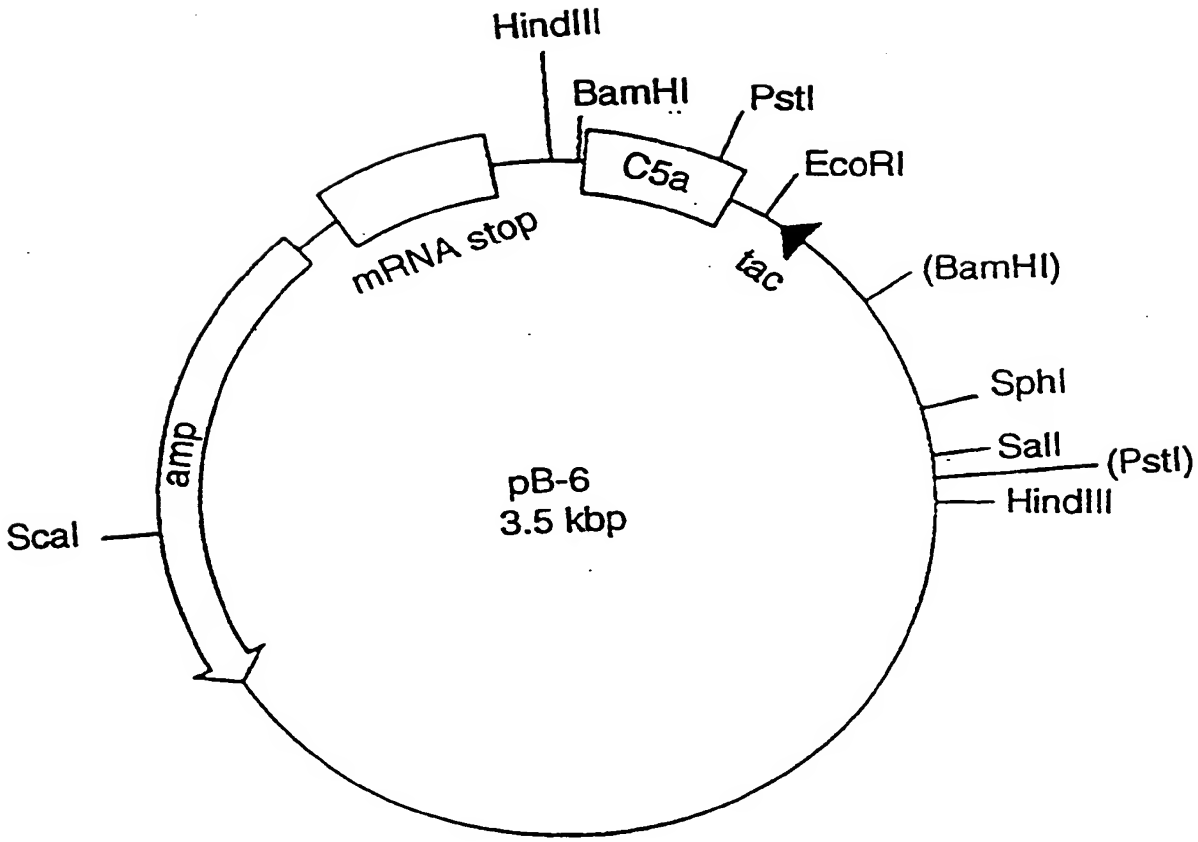
91. Use of a polypeptide derivative according to claim 1 or of a dimer according to claim 13 for the preparation of a pharmaceutical composition for treatment of a C5a-mediated disease or inflammatory condition in a mammal inclusive man.

Fig. 1



M13mp18/C5a

Fig. 2


HindIII
BamHI
PstI
EcoRI
C5a
mRNA stop
tac
(BamHI)
SphI
SalI
(PstI)
HindIII
amp
ScaI
pB-6
3.5 kbp

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/02422

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K16/18 A61K38/17 G01N33/68
C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 305 615 (IMMUNETECH PHARMACEUTICALS INC.) 8 March 1989 see the whole document, especially the sixth peptide of the structure list appearing on page 6 ---	1,13,14
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 82, no. 11, June 1985, WASHINGTON US, pages 3543-3547, XP002012988 W MANDECKI ET AL.: "Chemical synthesis of a gene encoding the human complement fragment C5a and its expression in Escherichia coli" see figure 1 --- -/--	1,13,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 September 1996	20.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/02422

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 220 864 (PFIZER INC.) 6 May 1987 see the whole document ---	1-91
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, no. 1, January 1989, WASHINGTON US, pages 292-296, XP002012989 K W MOLLISON ET AL.: "Identification of receptor-binding residues in the inflammatory complement protein C5a by site-directed mutagenesis" see the whole document ---	1-91
P,X	WO,A,95 16033 (CIBA-GEIGY AG) 15 June 1995 see the whole document ---	1-91
A	CHEMICAL ABSTRACTS, vol. 123, no. 21, 20 November 1995 Columbus, Ohio, US; abstract no. 277137c, I N BABKINA ET AL.: "Chemical-enzymic synthesis, cloning and expression of a gene coding for a human anaphylatoxin C5a analog " page 264; XP002012993 see abstract & BIOORG. KHIM., vol. 21, no. 5, May 1995, pages 259-364, -----	1-91

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